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Bap31 Enhances the Endoplasmic Reticulum Export and Quality Control of Human Class I MHC Molecules

John J. Ladasky,* Sarah Boyle,* Malini Seth,† Hewang Li,§ Tsvetelina Pentcheva,‖ Fumiyoshi Abe,¶ Steven J. Steinberg,¶ and Michael Edidin‡

The assembly of class I MHC molecules and their export from the endoplasmic reticulum (ER) is governed by chaperones and accessory proteins. We present evidence that the putative cargo receptor protein Bap31 participates in the transport and the quality control of human class I molecules. Transfection of the human adenocarcinoma cell line HeLa with yellow fluorescent protein-Bap31 chimeras increased surface levels of class I in a dose-dependent manner, by as much as 3.7-fold. The increase in surface class I resulted from an increase in the rate of export of newly synthesized class I molecules to the cell surface and from an increase in the stability of the exported molecules. We propose that Bap31 performs quality control on class I molecules in two distinct phases: first, by exporting peptide-loaded class I molecules to the ER/Golgi intermediate compartment, and second, by retrieving class I molecules that have lost peptides in the acidic post-ER environment. This function of Bap31 is conditional or redundant, because we find that Bap31 deficiency does not reduce surface class I levels. Overexpression of the Bap31 homolog, Bap29, decreases surface class I levels in HeLa, indicating that it does not substitute for Bap31. The Journal of Immunology, 2006, 177: 6172–6181.

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Abbreviations used in this paper: ER, endoplasmic reticulum; ERGIC, ER/Golgi intermediate compartment; CFFR, cystic fibrosis transmembrane conductance regulator; CADDIS, contiguous X-linked adrenoleukodystrophy/DX51357E deletion syndrome; X-ALD, X-linked adrenoleukodystrophy; siRNA, small interfering RNA; EGFP, enhanced GFP; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein.

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Molecules that contain the transmembrane sequence from the mouse IgG H chain are exported to the cell surface when expressed in insect cells without Ig~a~ and Ig~B~, but are retained when co-expressed with mouse Bap29 and Bap31 (19). Overexpression of wild-type Bap31 reduces the surface levels of the cystic fibrosis transmembrane conductance regulator (CFTR), a protein that is reported to misfold frequently; a malfunctioning CFTR mutant is retained more strongly still (20). Conversely, depletion of Bap31 increases surface levels of CFTR. Depletion of Bap31 also permits the cytochrome P450 2C2 protein to escape the ER and traffic to the nuclear membrane and the cell surface (21).

Here, we show that Bap31 is associated with human class I MHC molecules, and that overexpression of Bap31 increases the amount of class I on the cell surface, in a dose-dependent manner. The rate at which newly synthesized class I molecules reach the medial Golgi and the cell surface increases in the presence of excess Bap31. We also show that there is a higher fraction of long-lived class I molecules in cells that overexpress Bap31 than in control cells. The combination of a moderately increased export rate and increased stability of exported class I account for the increase in the steady-state levels of class I at the cell surface. However, neither small interfering RNA (siRNA)-mediated reduction of Bap31 levels, nor the loss of a functional BAP31 gene, affected class I levels or stability. Thus, Bap31 must be a conditional or redundant participant in the class I maturation pathway, rather than being an essential component. We have also found that the overexpression of Bap29 reduces surface class I levels, indicating that it does not substitute for Bap31.

Materials and Methods

Cell lines

HeLa cells were obtained from the American Type Culture Collection. The normal human fibroblast cell lines 049BB and 5659C were obtained from the Coriell Institute Cell Repository, CADDs4 and CADDs5 are two human fibroblast lines from patients with the contiguous X-linked adrenoleukodystrophy/DXS1357E deletion syndrome (CADDs) genotype (S. J. Steinberg, unpublished results), similar to the cell lines described in Ref. 22. X-linked adrenoleukodystrophy (X-ALD) 110362 and X-ALD 303617 are two human fibroblast cell lines from patients with ABCD1 gene mutations (23). These two cell lines are X-ALD (S. J. Steinberg, unpublished results), similar to the cell lines described in Ref. 23. The human Burkitt’s lymphoma cell line, Daudi, does not express class I MHC molecules on the cell surface, because it lacks a functional β2-microglobulin protein (24).

Cell cultures were maintained in a humid 5% CO2 atmosphere at 37°C, and were grown in RPMI 1640 (Daudi) or DMEM (HeLa and fibroblasts) containing 2 mM t-glutamine. Culture medium for HeLa and Daudi cells was supplemented with 10% heat-inactivated FBS. The medium for 5659C and 049BB cells was supplemented with 20% FBS (not heat-inactivated), 1% nonessential amino acid solution, and 1% vitamin solution. X-ALD and CADDs culture medium was supplemented with 10% FBS (not heat-inactivated), and an additional 2 mM t-glutamine for a total of 4 mM.

Construction of mammalian expression plasmids

Oligonucleotides were purchased from Integrated DNA Technologies. Total RNA was prepared from HeLa cells using TRIzol (Invitrogen Life Technologies). Oligonucleotides were purchased from Integrated DNA Technologies. To facilitate cloning and mammalian expression use, we have prepared the plasmid pN3-noGFP, a variant of the plasmid pEGFP-N3 (Clontech) from which we have deleted the enhanced GFP (EGFP) gene. pEGFP-N3 was cut with KpnI and BsrGI. The large fragment was isolated and self-ligated. The yellow fluorescent protein (YFP)-tapasin construct has been described previously (25). The pEYFP-N3 plasmid is a variant of pEGFP-N3 in which EGFP was replaced by EFY6 (p). The plasmid pA2-YFP-N3, which expresses a full-length human class I MHC, HLA-A2, fused to the YFP, was described in the same reference.

The chimera consisting of YFP fused to the transmembrane region and cytoplasmic tail of HLA-A2 was prepared from pA2-YFP-N3, and pGFP-gpi (26), which was kindly provided by S. Lacey (University of Texas Southwestern Medical Center, Dallas, TX). First, pA2-YFP-N3 was digested with BsiI; then, the single-stranded overhangs were removed with mung bean nuclelease; and finally, the product was digested with EcoRI. We retained the large fragment, which includes the C terminus of HLA-A2, up to the 10 aa immediately preceding the transmembrane region (EFSQQP TPI). The GFP-gpi plasmid was digested sequentially with BamHI, mung bean nuclease, and EcoRI. The small fragment from pGFP-gpi, containing the human folate receptor signal sequence, followed by the myc Ab epitope tag (EQKLISEEDL), and 11 aa from GFP (MSKGEELFTGV), was ligated to the large fragment from pA2-YFP-N3.

The BAP29-YFP expression plasmid was also prepared from pA2-YFP-N3. Fragments containing the entire leader–linker–transmembrane sequence from the human folate receptor (FKRT) and the cytoplasmic tail of HLA-A2, a 561-bp DNA fragment was prepared by PCR. This 561-bp fragment was used as a template, producing a 756-bp fragment carrying BAP29 with a deleted stop codon, and two silent mutations to remove internal restriction sites. Both the pA2-YFP-N3 plasmid and the 756-bp BAP29 fragment were cut with EcoRI and BamHI. The BAP29 fragment was ligated to the large plasmid fragment.

Primary BAP31 cDNA was prepared by RT-PCR, using the oligonucleotides BAP31F1 and BAP31R1. The YFP-BAP31 expression plasmid was assembled in several steps. PCR was performed using the primary BAP31 cDNA as a template. The nested PCR product and pN3-noGFP were both cut with BglII and NheI, and then ligated together. In this intermediate construct, BAP31 was inserted into pN3-noGFP in the antisense orientation. Error-free clones were identified by sequencing. A BamHI-BglII fragment containing YFP plus a 25-aa linker sequence, SSTMQGQMGQDM LYDDDDGDPAPPSG (27), was inserted into the BglII site of the pN3-BAP31 plasmid. A Salt-I-BsrGI fragment containing the folate receptor leader sequence, the myc tag, and most of the YFP sequence was ligated to the large Salt-I-BsrGI fragment of the pN3-YFP-BAP31 plasmid. Finally, the entire linker- myc-YFP-linker-BAP31 construct was excised from its plasmid with NheI and SalI, and then inserted in the sense orientation into pN3-noGFP, which had been cut with SalI and XhoI.

For the preparation of the BAP31-YFP expression plasmid, nested PCR was performed on the primary BAP31 cDNA. The nested PCR product and the pEYFP-N3 plasmid were both cut with EcoRI and NheI, and then ligated. Error-free clones were identified by sequencing. The sequence of the 18-aa linker between Bap31 and YFP in this construct, SGENSAVDG TAGPGSIAI, is mostly determined by the multiple cloning site of pEYFP-N3. The coding regions of all completed constructs were confirmed to be error-free by sequencing. Details of the primers for all the constructions are given by the authors.

Plasmid transfections

Approximately 5 × 10^6 HeLa cells were trypsinized, centrifuged, suspended in 0.50 ml of OptiMEM culture medium (Invitrogen Life Technologies) at 37°C containing 15 μg of plasmid DNA, and placed in a 4-mm gap electroporation cuvette. Cells were electroporated with a BTX model 600 electroporator (Harvard Apparatus) using the following settings: 240 V, 1500 μF, and 129 Ω. Cells were immediately diluted with 1.0 ml of DMEM plus 10% FBS at 37°C, and then diluted as desired for subsequent cell culture. Transient transfectants were typically analyzed 2 days after electroporation. Stable YFP/Bap31 and YFP-tapasin transfectants were obtained by culturing cells for ~3 wk in DMEM plus 10% FBS plus 0.60 mg/ml active G418 sulfate (Invitrogen Life Technologies). High expressors were enriched by fluorescence-activated cell sorting. Single-cell clones were prepared by limiting dilution. We selected three YFP-Bap31 clones, which expressed varying levels of the chimeric protein. In increasing order of expression (see Fig. 3A), these clones were named B8, C1, and E2. We only obtained one Bap31-YFP clone (1D5), which expressed comparably low levels of chimerica. Therefore, most of our studies focused on the YFP-Bap31 transfectants.

Antibodies

The rat anti-human Bap31 mAbs, CC-1 and CC-4 (28), were obtained from Affinity Bioreagents. Polyclonal rabbit antisera that recognizes the cytoplasmic tail of human Bap31 was prepared by SynPep. Rabbits were immunized with the peptide CLEEEAKLQAADVPMKDEE, conjugated via the N-terminal cysteine to a keyhole limpet hemocyanin carrier protein. With the exception of the initial cysteine residue, the peptide sequence is identical with the C terminus of the human Bap31 protein, and is known to contain the CC-1 epitope. In our hands, CC-1 and the rabbit antiserum were interchangeable (results not shown). Anti-Bap29 Ab was a gift from G. Shore (McGill University, Montreal, Canada).
The mouse mAb KE2 recognizes fully assembled human HLA-A, -B, and -C molecules, and is cross-blocked by the W6/32 Ab (29) (M. Edidin, unpublished results). The mouse mAb HC10 recognizes free human class I MHC H chains (30).

Anti-calnexin rabbit serum and anti-GFP mouse mAb were purchased from StressGen Biotechnologies. The mouse mAb G1/93, which recognizes ERGIC-53, was a generous gift from H.-P. Haeri (Biozentrum, University of Basel, Basel, Switzerland).

The anti-CD95 Ab MM 2/57 (Biosource), and the anti-CD95 Ab CH11 (Coulter Immunotech) were shared by B. Bochner (Johns Hopkins University School of Medicine, Baltimore, MD). A hybridoma expressing the anti-CD55 Ab MEM43, samples of the anti-CD59 Ab MEM118, and samples of the anti-CD147 Ab MEM M6/1 were all generous gifts from V. Horejsi (Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic). The mAb L5.1 recognizes the human transferrin receptor (31).

Direct fluorescent Ab conjugates were prepared using the FluoroLink Cy5-NHS ester conjugation kit (Amersham Biosciences), and the Alexa Fluor 546 mAb labeling kit (Molecular Probes). Donkey anti-rabbit Ig F(ab′)2, conjugated to Cy3 was purchased from Jackson ImmunoResearch.

Goat anti-mouse IgG and anti-mouse IgM antisera conjugated to Cy3, Cy5, and R-PE (R-PE) were purchased from Molecular Probes.

siRNA transfections

siRNA was purchased from Qiagen. The sequences will be provided by the authors on request. Transfections were performed using Oligofectamine (Invitrogen Life Technologies) as described previously (32). We administered a single 240-pmol dose of siRNA duplex to a 12-well dish of HeLa cells at 50% confluence. Maximal siRNA effects were observed 3 days after transfection. Transfection efficiencies of ~70% were achieved, as measured by flow cytometry.

Immunoprecipitation, Western blots, and endoglycosidase H assay

For the analysis of the coprecipitation of Bap31 and class I molecules (see Fig. 1), one protease inhibitor tablet (Roche Diagnostics) was added to each mL of 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 1 mM PMSF. Immunoprecipitations and Western blotting were otherwise performed as described previously (7). The endoglycosidase H assay for assessing the arrival of class I molecules at the medial Golgi was performed as described previously (7). Cells were pulsed for 20 min with medium containing [35S]labeled cysteine and methionine, and then chased for varying amounts of time with unlabeled amino acids. Class I molecules were precipitated from cell lysates using KE2. Proteins were resolved by SDS-PAGE using 10% acrylamide/bisacrylamide gel.

Flow cytometry

Fluorescence-activated cell sorting was performed on either an EPICS (Beckman Coulter) or a MoFlo (DakoCytomation) FACS. For analysis, we used a FACScalibur flow cytometer (BD Immunocytometry Systems) equipped with a 488-nm argon laser and a 647-nm diode laser. For most assays, cells were harvested with a PBS solution containing trypsin, collagenase, and EDTA. Trypsin was omitted when measuring surface CD71 levels, because the extracellular domain of CD71 is readily cleaved by trypsin (33).

We used 1–2 × 10^5 cells per sample. Each sample was stained with 2.5–5 μg of primary Ab in <100 μL. Secondary Ab, if needed, was added in kind. This ratio of Ab to Ag is higher than generally used in flow cytometry, and subjectively qualifies as “saturating” for several of the Abs we used, including KE2, CC-4, and KE2-Cy5 staining was recorded as the ratio between the fluorescence of the test cells and the reference cells. Using this approach, we reduced our reported coefficient of variance to 4% (data not shown). This ratio metric method has the added advantage that cell counts need not be equal between tubes to take a consistent measurement, allowing us to dispense with hemacytometer counting.

Dead cells were identified by their uptake of 7-aminocoumarin YC2 (Sigma-Aldrich) and were excluded from analysis.

Data analysis of the transfections in Fig. 7 was performed by converting flow cytometry list-mode data to a spreadsheet format using FCExtract version 1.02b by E. F. Glynn (Stowers Institute for Medical Research, Kansas City, MO). Graphs and least-squares regression fits were then prepared using OriginPro, version 6.1 (OriginLab).

Brefeldin A, measurement of surface class I stability

Cells at 50% confluence in 12-well dishes were incubated for up to 36 h in 0.75 ml of culture medium containing 5 μg/ml brefeldin A (Sigma-Aldrich). Cells were detached from culture dishes as described above, and then stained with KE2-Cy5. Surface class I levels were measured by flow cytometry. Untreated HeLa cells were used as a reference, as described above.

Papain, measurement of class I export

We modified a method for the depletion of class I molecules from the surface of living cells using papain (34). Briefly, a 10-cm dish of cells at 50% confluency was washed once with HBSS, and then overlaid with 2.5 ml of HBSS containing 2 mM L-cysteine, 1 mM EDTA, and 2 μM papain (Sigma-Aldrich). Cells were treated with papain for 3 h in a tissue culture incubator. Typically, cells were 60% viable after treatment, and surface class I levels were depleted 5-fold (data not shown). Following papain treatment, cells were washed twice with DMEM, returned to DMEM plus FBS, and cultured for up to 60 h. Cells were harvested and analyzed for surface class I as described for the brefeldin A assay, above.

Confocal fluorescence microscopy

Except as noted here, cells were prepared for intracellular staining as described previously (7). HeLa cells and Bap31 transfectants were used in most experiments. We also used X-ALD fibroblasts in some experiments, because they are very flat and well-spread, reducing artifacts from superposition of fluorescence from different volumes in the cytoplasm. Coverslips were fixed for 30 min at room temperature in PBS containing 4% paraformaldehyde (Electron Microscopy Sciences). Cells were washed with 0.25% NH4Cl in PBS, and then permeabilized with PBS containing 0.2% saponin and 1% BSA. Abs were diluted in permeabilization buffer. Anti-Bap31 serum was diluted 300×; CC-4 was diluted 500×; anti-calnexin serum was diluted 200×; and G1/93 was diluted 1000×. Fluorescent secondary Abs were diluted to 10 ng/μL. Before staining, Abs were airfuged at 120,000 × g for 1 h. Stained coverslips were protected from photobleaching using SlowFade mounting medium (Molecular Probes), and sealed to glass slides. Samples were imaged on a Zeiss LSM 510 confocal laser-scanning microscope, using the ×63 oil immersion lens. Images were acquired in multitrack mode to eliminate fluorescence cross talk.

Quantitative colocalization used the autothresholding algorithm of Costes et al. (35), as realized in the National Institutes of Health medical image processing, analysis, and visualization software package (http://www.nih.gov/ij/).

Results

Coprecipitation of human Bap31 and class I MHC

Recently, it was shown that mouse class I MHC molecules coprecipitate with Bap31 (7, 15). Human Bap31 also coprecipitates with fully assembled human class I MHC in HeLa cells (Fig. 1A), although most Bap31 molecules are not associated with class I MHC. Unlike mouse Bap31, human Bap31 also coprecipitates with free class I H chains. Some Bap31 also coprecipitates with free class I MHC H chains in the β2m-negative cell line, Daudi (Fig. 1A).

The transmembrane domain of the IgD molecule is sufficient to mediate the binding of IgD to Bap31 (36). We transfected cells with a plasmid expressing a tagged, truncated class I molecule consisting of YFP fused to the transmembrane and cytoplasmic domains of HLA-A2. Bap31 coprecipitates with this truncated class I molecule, as well as with a full-length HLA-A2-YFP chimera (Fig. 1B). The results imply that, as with IgD, Bap31 binds to the transmembrane region of class I H chains.

For subsequent studies, we constructed chimeric plasmids, comprising YFP fused to the N terminus or the C terminus of Bap31.
HeLa cells were transfected with \( \text{YFP-BAP31} \) or \( \text{BAP31-YFP} \). Both YFP-Bap31 and Bap31-YFP chimeric proteins coprecipitate with fully assembled class I molecules (Fig. 1C).

**Subcellular location of human Bap31**

We used qualitative and quantitative fluorescence microscopy to localize endogenous Bap31, YFP-Bap31, and Bap31-YFP in transfected cells. The pattern of YFP fluorescence of the transfectants was compared with that of endogenous Bap31 labeled with anti-Bap31 antiserum. The patterns of fluorescence were similar, although not identical, for all forms of Bap31. A significant fraction of Bap31 was found in the ER, as defined by colocalization with the ER marker, calnexin (Fig. 2, A–C). Colocalization between Bap31 and calnexin was extensive, but incomplete. Bap31 was often absent from peripheral, calnexin-rich regions of the cell. Also, Bap31 was enriched in a calnexin-poor, perinuclear region (Fig. 2C, arrowheads), which may include ERGIC and a novel ER-derived quality control compartment described recently (37).

**FIGURE 1.** Coimmunoprecipitation of Bap31 with class I MHC molecules. A, Western blotting of Bap31 with mAb CC-1. Fully assembled class I MHC from HeLa cells was serially immunoprecipitated with mAb KE2 (top row). Cleared supernatant (top row, right lane) possesses additional Bap31. Free class I H chains were serially immunoprecipitated with mAb HC10 (middle row); associated Bap31 was identified as for KE2. Bap31 also associates with free H chains in Daudi cells (bottom row). B, HeLa cells were transfected with constructs expressing the transmembrane and cytoplasmic domains of HLA-A2 fused to YFP (left), or with pA2-YFP-N3, which expresses the full-length HLA-A2 fused to YFP (right). YFP-tagged molecules were immunoprecipitated with anti-GFP Ab. Blots were probed with anti-GFP (top row, center row), or with CC-1 (bottom row). C, HeLa cells transfected with constructs expressing YFP-Bap31 (top row) or Bap31-YFP (bottom row). Class I was serially immunoprecipitated with KE2 and blots were probed with anti-GFP. Chimeric proteins run in proximity to a nonspecific band (*), which may be IgH; this band is absent from whole-cell lysate (data not shown) and cleared supernatant (right column).

**FIGURE 2.** Subcellular localization of Bap31 by confocal fluorescence microscopy. A and B, In HeLa cells, both anti-calnexin Ab staining (A) and YFP-Bap31 fluorescence (B) show a reticulated staining pattern characteristic of the ER. C, Overlay of A and B show that colocalization of calnexin and Bap31 is extensive but incomplete, with Bap31 enriched in a calnexin-poor, perinuclear region. D–F, In HeLa, anti-ERGIC-53 staining (D) and YFP-Bap31 fluorescence (E) are not strongly correlated by eye (F), but the correlation is apparent on two-dimensional fluorescence correlation histogram (inset) (35). In this histogram, red and green pixel intensity are plotted on horizontal and vertical axes, respectively; least-squares-fit (diagonal yellow line) is used to search for a pair of thresholds (yellow horizontal and vertical lines) that intersect at the diagonal and that lie just above the subset of data for which the correlation coefficient is zero. G–J, Same as D–F, respectively, but with X-ALD fibroblast cells. Correlation coefficients and percentage colocalization are given in Table I, along with values for negative and positive controls.
This localization was not an artifact of the YFP tag, because endogenous Bap31, visualized with Ab, was also found in this region (data not shown). Similar colocalization patterns were observed between Bap31 and protein disulfide isomerase, another ER marker (Ref. 12 and data not shown). Bap31 clearly did not colocalize with giantin or with mannosidase II, two markers for the Golgi complex (data not shown).

In HeLa cells and fibroblasts cultured under normal conditions, colocalization between ERGIC-53 (Fig. 2, D and G) and Bap31 (E and H), read as yellow fluorescence, was not immediately apparent by eye (F and J). A recently developed quantitative colocalization algorithm (35) confirmed that some Bap31 colocalizes with ERGIC-53. Results of the quantitation are summarized in Table I. The correlation coefficients (r) for Bap31 and ERGIC-53 are nearly as high as that determined for HLA-A2 molecules labeled with both cyan fluorescent protein (CFP) and YFP in tandem (6), and significantly higher than the correlation observed between Bap31 and giantin, or Bap31 and mannosidase II. The fluorescence correlation results did not depend on the method that we used to label Bap31 (not shown).

Our findings agree with those of Annaert et al. (12), who identified a fraction of hamster Bap31 in a perinuclear, post-ER compartment by microscopy. Furthermore, in their hands, density centrifugation yielded a cell fraction that contained significant amounts of both Bap31 and ERGIC-53.

### Table I. Quantitative correlation fluorescence microscopy (33) of Bap31 vs ERGIC-53

<table>
<thead>
<tr>
<th>Cells</th>
<th>Labeled Proteins</th>
<th>Correlation Coefficient (r)</th>
<th>Percent ERGIC Colocalized with Bap31 (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Bap31, anti-giantin; Bap31, anti-mannosidase II</td>
<td>0.35</td>
<td>N/A</td>
<td>Negative control, minimally correlated</td>
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<td></td>
<td>HLA-A2 CFP-YFP tandem</td>
<td>0.93 ± 0.03</td>
<td>N/A</td>
<td>Positive control, maximally correlated</td>
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<td></td>
<td>Anti-Bap31, anti-ERGIC-53</td>
<td>0.81 ± 0.01</td>
<td>60</td>
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<td>X-ALD</td>
<td>Anti-Bap31, anti-ERGIC-53</td>
<td>0.90 ± 0.01</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

*The correlation coefficient (r) is for the subset of data that lies above automatically defined thresholds (see Fig. 2, insets).

Bap31 overexpression specifically increases surface class I levels

Quantitative measurements of mature surface class I molecules on HeLa cells and transfectants were performed by flow cytometry, using a universal anti-class I MHC Ab, KE2, conjugated to Cy5. Bap31 expression levels were assessed by YFP fluorescence. The only stable BAP31-YFP transfectant that we obtained was dim, and did not measurably increase surface class I. However, transient transfection of BAP31-YFP did increase surface class I levels, in proportion to the amount of Bap31 expressed (see Fig. 7C). We obtained several stable clones that expressed YFP-Bap31 to different extents. Surface class I was increased in these clones with YFP-Bap31 expression, in a dose-dependent manner (p < 0.05) (Fig. 3, A and B). In clone E2, which expresses the most YFP-Bap31, we measured a 3.7-fold increase in surface class I levels, relative to untransfected HeLa cells.

To assess the breadth of the effect of Bap31 overexpression, we compared the expression levels of other surface proteins on HeLa...
cells and on the YFP-Bap31 transfectants. Bap31 does not appear to induce a general up-regulation of HeLa cell surface proteins. None of the six proteins—CD9, CD55, CD59, CD71, CD95, and CD147—were affected by overexpression of Bap31 (Fig. 3, C–H). Our results agree with a report that the trafficking of CD71 is unaffected by Bap31 (12), but contrast somewhat with a report that deletion of the BAP31 gene, or caspase cleavage of Bap31, can impair the export of CD9 in mouse embryonic stem cells (38).

As a negative control, we transfected HeLa cells with free YFP (Fig. 3, A and B). The surface levels of class I were unchanged in these cells, confirming that the increase in surface class I observed in the YFP-Bap31 transfectants was caused by the Bap31 portion of the chimeric protein. Stable HeLa transfectants expressing YFP-tapasin (25) were also prepared, and were intended to serve as a second negative control. In contrast to a previous report that the overexpression of tapasin in HeLa does not increase surface class I levels (39), we observed a 2-fold increase in our YFP-tapasin transfectant.

We were not able to isolate stable clones of HeLa transfected with either YFP-Bap29 or Bap29-YFP.

**Bap31 overexpression increases the export rate of nascent class I MHC molecules**

If Bap31 functions as a cargo receptor for class I MHC proteins in the ER, and is normally limiting, then the rate at which nascent class I molecules are exported to the medial Golgi should increase in cells expressing excess Bap31. This was the case. The rate of traffic of newly synthesized class I molecules to the medial Golgi, measured in terms of maturation of oligosaccharides to endo H resistance, was 24 ± 6 min (n = 4) in untransfected HeLa cells. The rate of maturation increased in proportion to the level of Bap31 expressed, up to 1.5-fold over controls (Fig. 4, A–C).

A 1.5-fold increase in the rate of ER-to-Golgi transport is inadequate to explain the 3.7-fold increase in the steady-state surface levels of class I. In considering this discrepancy, we had two concerns about the pulse-chase assay: first, that our autoradiography was insufficiently quantitative; second, that the manipulations required for the pulse-chase assay delayed the maturation of class I. To address these concerns, we depleted class I molecules from the surface of HeLa cells and Bap31 transfectants by papain digestion, and then monitored class I recovery by flow cytometry (Fig. 4, D and E). Recovery rates were computed from first-order exponential fits to the data, normalized to an untransfected HeLa cell reference (Fig. 4D). Recovery rates were computed from first-order exponential fits to the data, normalized to an untransfected HeLa cell reference (Fig. 4D). Recovery rates were computed from first-order exponential fits to the data, normalized to an untransfected HeLa cell reference (Fig. 4D).

**Overexpression of Bap31 increases the stability of surface class I**

The steady-state level of a surface protein is determined by a balance between the rate of delivery of newly synthesized molecules to the cell surface, and the rate of loss of resident surface molecules by shedding, endocytosis, or other processes. To assess the effect of Bap31 on the lifetime of class I molecules on the cell surface, we blocked the secretory pathway for varying lengths of time using brefeldin A (40), and then measured surface class I levels by flow cytometry.

Class I molecules disappear more quickly from the surface of untransfected HeLa cells than from HeLa cells overexpressing either YFP-Bap31 or a positive-control, YFP-tapasin. Single-exponential fits to the data (Fig. 5A, thin line) consistently underestimated early data points, and overestimated middle-to-late data points. Biexponential fits yielded much better approximations to the data (Fig. 5, A and B). The rate at which class I reappeared on the cell surface increased with increasing expression of Bap31. In clone E2, which expresses the most YFP-Bap31, we observe a 2.2-fold increase in the export rate. This increase is comparable to the increase in the rate at which nascent class I molecules acquire endo H resistance. However, it still cannot account for the increase in steady-state surface class I levels induced by the overexpression of YFP-Bap31.

**Overexpression of Bap31 accelerates the maturation of class I MHC**

HeLa cells and YFP-Bap31 transfectants were pulsed with [35S]Cys plus Met, chased with unlabeled amino acids for 0–120 min, lysed, and immunoprecipitated with the anti-MHC Ab KE2. Half of each sample was treated with endoglycosidase H, so that nascent, endo H-sensitive (endoS) class I, which has not reached the medial Golgi was distinguishable from more mature, endo H-resistant class I A. Typical autoradiograms. Untransfected HeLa cells and YFP-Bap31 clone E2 are shown. Four bands are observed in lanes treated with endo H. Lower two bands, endo H-sensitive (endoS) class I; upper two bands, endo H-resistant (endoR). B, Maturation rates computed from quantitative densitometry of autoradiograms in A. First-order exponential curves were fit to the data. EndoS-class I in untransfected HeLa (○, thin line) has a half-life of 20.1 min; in clone E2 (○, thick line), 13.4 min. C, Summary of maturation rates measured in all pulse-chase experiments, normalized to maturation rate of untransfected HeLa cells. ○ and □, rates computed from experiments performed on two separate days. Height of solid bar indicates mean relative maturation rate. D, Effect of Bap31 overexpression on reappearance of class I molecules after stripping surface class I with papain. First-order exponential curves were fit to the data. One set of measurements from untransfected HeLa (○, thin line) and one from clone E2 (○, thick line) are shown. E, Summary of class I recovery rates measured after papain treatment, normalized to rate of recovery in untransfected HeLa cells. Circles, squares, and solid bars are as described for C. Both YFP-Bap31 and YFP-tapasin transfectants export class I molecules to the cell surface more rapidly than untransfected HeLa.
population (which we designate here as \( t_1 \)); the half-life of the long-lived, more stable population (\( t_2 \)); and the ratio of the number of molecules in the long-lived population to the number in the short-lived population (\( r \)). To characterize the increase in surface class I stability in greater detail, we examined each of the three variables separately (Fig. 5, B–D). In untransfected HeLa, \( t_1 = 4.1 \) h, and \( t_2 = 33 \) h. Long-lived class I molecules slightly outnumber their unstable counterparts, \( r = 1.1 \). These results are comparable with those reported for the stability of H-2Kb molecules on mouse lymphocytes (42). With the possible exception of clone E2, which appears to exhibit somewhat longer half-lives for each population (\( t_1 \) and \( t_2 \)), no trend is observed in \( t_1 \) or \( t_2 \) as a function of the amount of Bap31. However, a consistent, dose-dependent response is observed in \( r \) (p < 0.05).

In clone E2, which expresses the most YFP-Bap31, there are twice as many stable class I molecules as there are unstable molecules, \( r = 2.0 \). HeLa cells that express YFP-tapasin also present twice as many stable class I molecules as unstable molecules. There is no apparent change in \( t_1 \) or \( t_2 \).

**Bap31 deficiency does not reduce levels of surface class I**

Because the overexpression of Bap31 increases surface class I levels, we investigated whether depletion of Bap31 would reduce surface class I by transfecting cells with siRNA. We prepared siRNA duplexes that correspond to human BAP31 cDNA and, as a positive control for class I loss, human B2M cDNA (24). A nonsense siRNA, which corresponds to no known human cDNA sequence, served as a negative control. The efficacy of the BAP31 siRNA was confirmed by the reduction of fluorescence (3.5-fold) from cells expressing YFP-Bap31 (Fig. 6A). The B2M siRNA reduced surface class I levels 5-fold (Fig. 6B). However, cells that had been transfected with the BAP31 siRNA showed no change in surface class I levels when compared with the fraction of cells in the B2M siRNA treatment that remained untransfected (Fig. 6B), or when compared with cells treated with the nonsense siRNA control (data not shown).

Humans with a natural deletion across the BAP31 locus (also known by its genetic designation, DXS1357E) have been recently

**FIGURE 5.** Bap31 overexpression increases the stability of surface class I MHC molecules. Export of nascent class I to the cell surface was halted by culturing cells in medium containing brefeldin A. Surface class I loss was monitored over a 36-h period by flow cytometry using KE2-Cy5. A. Loss of surface class I as a function of time in control, untransfected HeLa cells. The curves shown are the best first-order exponential decay fit (thin line) and the best second-order fit (thick line). The equation for the second-order curve fit describing the surface concentration of class I MHC (\( y \)) as a function of time (\( t \)) is \( y = x_1 e^{-t_1} + x_2 e^{-t_2} \). B–D, Half-life (mean ± SEM) of the unstable class I population (\( t_1 \)) (B), half-life of the stable class I (\( t_2 \)) (C), and the ratio of stable to unstable class I (\( r = x_1/x_2 \)) (D).

**FIGURE 6.** Effect of Bap31 depletion on steady-state levels and stability of surface class I MHC molecules. A, YFP-Bap31 clone B8 was treated with siRNA against BAP31 (black line) or with a nonsense siRNA (gray fill). Loss of yellow fluorescence shows the efficacy of the BAP31 siRNA. B, siRNA depletion of BAP31 to approximately one-third of steady-state level did not reduce surface class I levels (black line). siRNA against B2M is a positive control for class I depletion, with ~70% of HeLa cells showing a 5-fold reduction in surface class I levels (gray). Surface class I levels on untreated HeLa cells, and on HeLa treated with a nonsense siRNA control (not shown) were identical with those obtained with the BAP31 siRNA. C, Normalized steady-state surface class I levels on wild-type human fibroblasts 0498B and 5659C, X-ALD fibroblasts 110362 and 303617, and CADDS fibroblasts CADDS4 and CADDS5.
HeLa (half-life of the stable fraction of class I generally exceeded that of plasmids. of equal amounts of BAP29-YFP.

Transient transfection of Bap31-YFP. Expansion of the selected region from A; least-squares linear regression fit shows a negative correlation between surface class I levels and Bap29-YFP expression. C. Transient transfection of Bap31-YFP. D. Transfection of equal amounts of BAP29-YFP and BAP31-YFP plasmids.

Overexpression of Bap29 reduces surface levels of class I

Because Bap31 deficiency does not reduce surface class I levels (Fig. 6), we considered the possibility that Bap31 is redundant with Bap29 in the class I maturation pathway. Western blotting indicated that the Bap31 homolog, Bap29, is found in CADDS cells at levels comparable with those in wild-type cells (data not shown). Deletion of both BAP29 and BAP31 in mouse embryonic stem cells was reported to impair the export of class I (15). HeLa cells were transfected with Bap29-YFP, and changes in surface class I MHC levels were assessed. We were surprised to observe that surface class I levels decreased in proportion to the level of Bap29-YFP expression (Fig. 7, A and B). Bap31-YFP transfection increased surface class I MHC levels (Fig. 7C), and its effect was codominant with the effect of Bap29-YFP; in cells expressing both plasmids, there was no change in surface class I levels with increasing YFP expression (D). Transfection of free cytosolic YFP into HeLa had no effect on surface class I levels (Fig. 3). Finally, we found that surface class I levels in CADDS5 cells were not affected by the transient transfection of Bap29-YFP, Bap31-YFP, or free YFP (data not shown).

Discussion

Recent findings indicate that class I molecules pass through additional intracellular regulatory steps, subsequent to dissociating from Tap. Fully assembled class I molecules are not immediately exported from the ER (5, 6). Providing an excess of high-affinity peptide accelerates the dissociation of class I molecules from the Tap complex, but still has no effect on the rate at which class I progresses to the medial Golgi (7). These findings imply that an active process is required to export fully assembled class I molecules from the ER. We recently reported that class I coprecipitates with Bap31 (7), a putative anterograde cargo receptor for cellubrevin (12) and for mannosidase II (9). Subsequently, Paquet et al. (15) have reported that the simultaneous deletion of BAP31 and BAP29, slightly impairs the export of class I from the ER.

The central finding in our present report is that overexpression of Bap31 increases the amount of fully assembled class I MHC on the cell surface. This is due to effects of Bap31 on the rate of forward traffic of class I MHC molecules, and on the stability of these molecules at the cell surface. The effect on anterograde traffic

FIGURE 7. Bap29-YFP expression reduces surface class I levels. A, HeLa cells were transiently transfected with Bap29-YFP and analyzed by two-color flow cytometry. Transfected cells were identified by high levels of yellow fluorescence (rectangle, upper right). B. Expansion of the selected region from A; least-squares linear regression fit shows a negative correlation between surface class I levels and Bap29-YFP expression. C. Transient transfection of Bap31-YFP. D. Transfection of equal amounts of BAP29-YFP and BAP31-YFP plasmids.
is consistent with a function for Bap31 in exporting newly synthesized class I MHC molecules from the ER.

The effect of Bap31 on the lifetime and stability of surface class I is consistent with a role in quality control—for example, promoting the binding of high-affinity peptides to nascent class I molecules (41). The free class I H chains that we found in association with Bap31 are not necessarily all newly synthesized molecules. Instead, these free H chains may be components of previously assembled class I that have lost their peptides, triggering a concurrent dissociation from $\beta_{2m}$ (46–48). The dissociation of $\beta_{2m}$ triggers conformational changes across the transmembrane region of the MHC H chain, which is the putative site of Bap31 binding, and into the cytoplasmic tail (48, 49). Bap31 may therefore function in quality control by acting as a retrograde transporter, retrieving class I molecules that have lost their peptides in post-ER compartments. The acidic pH of the secretory pathway could play a role in destabilizing peptide-MHC bonds (50, 51). Retrieval of class I molecules would be mediated by the binding of Bap31’s C-terminal dilysine motif to COP I. Bap31 has been shown to prevent the export of unassembled IgD molecules (19) and misfolded CFTR proteins (20), processes that could also, in principle, involve retrograde transport.

A similar quality-control mechanism has been proposed for HLA-G, a nonclassical human class I MHC molecule with limited expression (52). Unlike classical class I molecules, HLA-G possesses a C-terminal dilysine motif that could bind COP I directly. HLA-G’s dilysine motif has been demonstrated to function in the retrieval of HLA-G molecules that were bound to lower-affinity antigenic peptides. HLA-G’s quality control mechanism would presumably be functional in the absence of Bap31, but the purpose and the method of retrieval are the same as what we propose here for classical class I.

Whatever the underlying mechanism, our results show that Bap31 is a limiting factor in the maturation and traffic of the class I MHC molecules of HeLa cells. Despite this, it is not essential. Reduction of Bap31 levels to roughly one-third of steady-state levels with siRNA did not affect levels of class I molecules on the surface of HeLa cells; class I molecules of cells lacking a functional BAP31 gene appeared normal in amount and stability. We also investigated the possibility that the Bap31 homolog, Bap29, provides a redundant function in the class I export pathway. Overexpression of Bap29 in HeLa cells decreased surface levels of class I, rather than enhancing them. Our results exclude the possibility that Bap29 can substitute for Bap31 in every circumstance, and raise the possibility that Bap29 can function as a negative regulator of class I surface expression. Our results also appear to contrast with the finding that deletion of both BAP29 and BAP31 in differentiated mouse ES cells modestly decreased the amount of class I that colocalizes with COP II, and retarded the transport of class I MHC molecules into the medial Golgi (15). Finally, overexpression of Bap29 in human CADD8 cells did not decrease class I levels as we observed in HeLa. Any effect that Bap29 may have on the class I pathway may therefore depend on the presence of Bap31.

In parallel to our findings for Bap31, we showed that overexpression of YFP-tapasin causes an increase in surface class I levels, rate of export, and lifetime. Like Bap29 and Bap31, tapasin possesses a C-terminal dilysine retrieval motif. Taken alone, the results in the present report might suggest that tapasin, rather than Bap29, functions as a redundant cargo receptor for class I (53). However, our results can also be explained by other well-established functions for tapasin (reviewed in Ref. 54), such as the promotion of a peptide-receptive conformation in class I molecules; recruitment of class I to the TAP complex; stabilizing the expression of Tap; and promoting peptide translocation by Tap. Reports from our laboratory (6) and others (53) disagree as to whether tapasin can be found in post-ER compartments. Tapasin’s dilysine retrieval motif is at least partially dispensable to tapasin’s role in class I maturation (55). The extent to which class I molecules require the presence of tapasin depends upon polymorphisms in class I H chain sequences (43–45). Although Tap and tapasin greatly facilitate the loading of class I molecules with peptides, some class I molecules can acquire peptides in their absence. Similarly, it is possible that only certain alleles of class I, or certain specific unfolded states, require Bap31 or Bap29 for quality control. Therefore, the functional overlap between Bap31, Bap29, and other potential cargo receptors in the class I export pathway may be incomplete.

Similar questions about nonessential, partially overlapping, and antagonistic functions have arisen in studies of the YET genes, homologs of the mammalian BAP genes found in Saccharomyces cerevisiae (56) (V. Goh, M. Edidin, and K. Cunningham, unpublished results). No essential function was impaired by the deletion of YET1, YET2, YET3, or combinations thereof. Disruption of YET1 moderately accelerated yeast cell growth in liquid culture, whereas disruption of YET3 exhibited slower growth and reduced secretion of the invertase enzyme. A YET1-YET3 double mutant returned to normal growth rates. The redundancy of proteins in the Bap family remains a paradox.

The fate of class I H chains that might be retrieved from post-ER compartments is unknown. The class I assembly pathway in HeLa cells is not saturated with H chains, as evidenced by the fact that class I H chain overexpression increases Ag presentation far more readily than the overexpression of $\beta_{2m}$, Tap, or tapasin (40). Although many proteins that fail a quality-control step are exported to the cytosol for degradation (57), it is attractive to speculate that retrieved class I H chains might be recovered, even if the peptides to which they were initially bound were labile. Recycling these H chains, if possible, would be an efficient use of an unsaturated class I Ag presentation system.

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Disclosures

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