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Endoplasmic Reticulum Targeting Alters Regulation of Expression and Antigen Presentation of Proinsulin

Hsiang-Ting Hsu,*,†,1 Linda Janßen,*,†,‡ Myriam Lawand,*,†,‡ Jessica Kim,*,†,‡ Alicia Perez-Arroyo,*,†,‡ Slobodan Culina,*,†,‡ Abdel Gdoura,*,†,‡ Anne Burgevin,*,†,‡ Delphine Cumenal,*,†,‡ Yousra Fourneau,*,†,‡ Anna Moser,*,†,‡ Roland Kratzer,*,†,‡ F. Susan Wong,*, Sebastian Springer,§ and Peter van Endert*,†,‡

Peptide ligands presented by MHC class I (MHC-I) molecules are produced by degradation of cytosolic and nuclear, but also endoplasmic reticulum (ER)–resident, proteins by the proteasome. However, Ag processing of ER proteins remains little characterized. Studying processing and presentation of proinsulin, which plays a pivotal role in autoimmune diabetes, we found that targeting to the ER has profound effects not only on how proinsulin is degraded, but also on regulation of its cellular levels. While proteasome inhibition inhibited degradation and presentation of cytosolic proinsulin, as expected, it reduced the abundance of ER-targeted proinsulin. This targeting and protein modifications modifying protein half-life also had profound effects on MHC-I presentation and proteolytic processing of proinsulin. Thus, presentation of stable luminal forms was inefficient but enhanced by proteasome inhibition, whereas that of unstable luminal forms and of a cytosolic form were more efficient and compromised by proteasome inhibitors. Distinct stability of peptide MHC complexes produced from cytosolic and luminal proinsulin suggests that different proteolytic activities process the two Ag forms. Thus, both structural features and subcellular targeting of Ags can have strong effects on the processing pathways engaged by MHC-I–restricted Ags, and on the efficiency and regulation of their presentation. The Journal of Immunology, 2014, 192: 4957–4966.

Selection, recruitment, and processing of cellular Ags broken down to peptides presented by MHC class I (MHC-I) molecules have been studied extensively in the last 15 y (1). After the initial demonstration that proteasome inhibitors reduced peptide supply to MHC-I molecules dramatically (2), a vast body of evidence in support of a central role of this large cytosolic protease in the breakdown of class I–presented Ags has been accumulated (3). However, although a general-purpose backup proteolytic system probably does not exist, presentation of some epitopes and peptide loading of some MHC-I alleles is not affected, or even enhanced, in the presence of proteasome inhibitors (reviewed in Ref. 4).

For a number of epitopes, such “proteasome–independent” presentation has been shown to involve tripeptidyl peptidase II (TPPII), another large cytosolic protease (reviewed in Ref. 5), and, more recently, insulin-degrading enzyme (6). Partial proteasome inhibition that favors cleavage at the epitope C terminus whereas inhibiting destructive internal cleavage is a second mechanism likely to underlie some cases in which presentation is enhanced or unchanged in the presence of proteasome inhibitors (7). However, these mechanisms may not be sufficient to explain the number of MHC-I ligands produced in a proteasome-independent manner, and additional mechanisms may remain to be discovered. Moreover, considering the strong effects of proteasome inhibition on numerous cellular pathways (8), inhibition of epitope presentation by proteasome inhibitors is not sufficient to prove that the source Ag is indeed broken down by the proteasome, so that the number of epitopes produced by the proteasome may be overestimated.

Recent studies have provided strong evidence that many MHC-I ligands are derived from rapidly degraded proteins (9). Many of these presumably correspond to defective ribosomal products that fail to become functional proteins because of incomplete synthesis, folding, posttranslational processing, or assembly. Current concepts hold that cytosolic, nuclear, and endoplasmic reticulum (ER)–targeted proteins are indiscriminately recruited for production of MHC-I ligands. Recruitment of the latter proteins involves the ER-associated degradation (ERAD) pathway through which proteins unable to pass the ER quality-control checkpoints are retrotranslocated into the cytosol for proteolysis by the proteasome (10). However, the nature and stringency of a possible link between ER quality control and production of MHC-I ligands remain unclear. Although one study has demonstrated a correlation between the folding capacity available in the ER and production of an MHC-I ligand (11), other studies have suggested that alterations such as removal of N-glycosylation sites or disulphide bridges, which both perturb protein stability and folding, do not affect Ag presentation (12).

Insulin, a hormone derived from ER-targeted proproinsulin (PPI) by sequential proteolytic processing in pancreatic β cells, qualifies

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as an interesting example of a donor of MHC-I ligands in light of several considerations. Insulin is a critical autoantigen in human type 1 diabetes (T1D), as well as in the principal rodent model of T1D, the NOD mouse, in which insulin appears to trigger autoimmunity (13). In NOD mice, a significant population of CD4+ and CD8+ T cells found in early islet infiltrates recognizes insulin, and both cell types can transfer disease. Insulin-specific autoreactive T cells are also detected in the blood of T1D patients (14), and insulin-specific CD8+ T cells can kill β cells in humanized NOD mice (15).

Given that insulin is expressed in thymic epithelial cells and that insulin is constantly present in the peripheral circulation, autoreactive T cells that recognize insulin should be exposed to strong pressure resulting in negative selection or induction of peripheral tolerance (13). Indeed, immunodominant CD4+ and CD4+ T cell insulin epitopes display low MHC binding affinity, resulting in low-avidity interactions between peptide–MHC complexes and T cells (16). As noted by other authors, it is surprising that such low-avidity interactions are sufficient to trigger autoimmunity. One possibility, proposed by Suri et al. (17), is that very high concentrations of β cell proteins or debris are released during T1D pathogenesis, thereby both overcoming low T cell avidity and explaining the tissue specificity in the face of systemic presence of soluble insulin. Another possibility relates to the exclusive production of PPI, comprising a leader and the C peptide in addition to insulin, by β cells (13). However, the immunodominant CD4+ and CD8+ T cell epitopes reside in the insulin B chain, which argues against this hypothesis. Alternatively, Ag processing of ER-targeted PPI in β cells may differ from that of internalized insulin, resulting in quantitatively or qualitatively distinct presentation of insulin epitopes. The fact that β cells display a constitutive activation of the unfolded protein response (UPR), a tripartite cellular response to high protein load in the ER that includes upregulation of the ERAD pathway, might also be of interest in this context (18).

We have studied the processing and Ag presentation of insulin, and we report that ER targeting has a strong effect both on the expression of insulin in the presence of proteasome inhibition and on the proteolytic pathways involved in its processing for MHC-I presentation. These results contribute to our understanding of the processing of ER-targeted Ags, but they may also be of relevance for the role of insulin as primary autoantigen in T1D.

Materials and Methods

Vaccinia viruses

Recombinant vaccinia viruses expressing human PPI and OVA have been described previously (19) and were produced in HeLaS3 cells by standard procedures. Considering a published report that proteasome inhibitors prevent expression of late vaccinia-encoded proteins (20), we used a synthetic early/late promoter contained in the pSC65 plasmid to produce the PPI virus. A total of $5 \times 10^7$ HeLa tet-on (HT)-Kd cells (see later) were seeded in six-well plates on day −1. The next day, the cells were treated with or without 0.5 μM epoxomicin (Epo) for 2 h followed by virus infection at a multiplicity of infection of 2 in DMEM with 2.5% FBS for 2 h. Then the medium was replaced with fresh complete DMEM with or without the drug, and the incubated for another 2 h, harvested, and analyzed by immunoblot.

HeLa Tet-on-Kd cells

An HT cell line expressing the reverse tetracycline-controlled transactivator was purchased from Clontech (Mountain View, CA). HT cells were cultivated in DMEM (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Hyclone, South Logan, UT), 2 mM t-glutamine (PAA, Pasching, Austria), 1% MEM nonessential amino acids (Life Technologies, Invitrogen, Carlsbad, CA), and 200 μg/ml Geneticin-418 (PAA). HT cells expressing the mouse class I molecules H2-Kd or H2-Kb were produced by transfection with pcDNA3.1/hygro(-) plasmids (Invitrogen, Cergy Pontoise, France) containing the corresponding full-length cDNAs that had been cloned from bulk splenocyte cDNA by high-fidelity PCR, using primers containing Xbal (5’) and BamHI (3’) restriction sites. Transfectants were selected with 0.4 μg/ml hygromycin B, cloned with cloning cylinders, and examined for MHC-I expression by flow cytometry. For each class I molecule, one clone displaying high-level monolocal class I expression was selected for further experimentation.

Reagents

Epo (0.15–0.5 μM), MG-132 (2 μM), AAF-CMK (20–50 μM), and bafilomycin A1 (50 μM) were purchased from BioMol (London, U.K.); lactacystin (10 μM) was purchased from Calbiochem (San Diego, CA). Tunicamycin (5 μg/ml), brefeldin A (10 μg/ml), and cycloheximide (CHX; 20 μg/ml) were purchased from Sigma (St. Louis, MO). Buthionamide (250 μM) was purchased from Tocris (Bristol, U.K.) and added each 90–120 min following published protocols (21). The inhibitor concentrations used are indicated in parentheses.

Abs

Anti-insulin Ab (H-86) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-β2-microglobulin was from Dako (Trappes, France). Secondary Abs for immunoblots were goat anti-rabbit HRP and goat anti-mouse HRP (Jackson ImmunoResearch Laboratory, Suffolk, U.K.) used at 1:25,000. Anti–CD8-PE and anti–IFN-γ-PE Abs were from BD Biosciences (Rockville, MD). Expression of H-2Kd was examined using Ab 20-8-4S.

pTRE-Tight constructs

PPI expression plasmids were constructed in the vector pTRE-Tight (pT; Clontech) containing the Tet Response Element. Initially, the pT cloning site was modified by insertion between the SalI and XbaI sites of complementary oligonucleotides encoding a hemagglutinin tag YPYDVPDYA, or the OVA CDS T cell epitope SIFNEKL. Then cDNAs encoding PI or PPI were amplified from a previously described cloned human PPI sequence (19), using primers containing BamHI (5’) and SalI (3’) sites, and subcloned into modified or unmodified pT. Plasmids expressing PPI in which one or several Cys residues were replaced by Ser were constructed by site-directed mutagenesis using the QuikChange kit (Strategene, La Jolla, CA) and Phusion high-fidelity polymerase (New England Biolabs, Beverly, Mass.; New England Biolabs). Abs were confirmed by sequencing.

Transient transfection

HT-Kd cells were transfected with pT plasmids using a Gene Pulser Xcell electroporator (Bio-Rad, Hercules, CA). A total of $6 \times 10^5$ cells in 250 μl electroporation buffer, 10 mM HEPES in PBS, were electroporated with 20 μg endotoxin-free DNA at 250 V and 900 μF. Pulsed cells were recovered in warm DMEM with 20% FBS. Protein expression was induced by addition of 0.5–1.0 μg/ml doxycycline (Doxxy; Sigma) either immediately or 16–18 h after transfection, as indicated. Where indicated, cells were treated with drugs before, during, and/or after induction of protein expression. Cells were harvested using trypsin, washed once in PBS with 1 mM PMSF (Sigma), counted, and analyzed by immunoblot.

Immunoblot

Cell pellets were resuspended in loading buffer with 100 mM DTT at a concentration of $1 \times 10^7$ cells/ml. Ten microliters of cell lysate was loaded on 12.5% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes, blocked with 5% skim milk, and stained with anti-insulin Abs used at 260 ng/ml. Abs to β2-microglobulin or β-actin (both used at 1:5,000) were used as loading controls. HRP-coupled secondary Abs were used at 1:25,000. Loading controls were stained on the same membrane as PPI. Images were taken with a CCD camera (Fujifilm, Tokyo, Japan).

Microscopy

HT cells were electroporated with plasmids encoding PPI or PI. Cells were seeded on coverslips in a 24-well plate, and protein expression was induced immediately with 1 μg/ml Doxxy. After 24 h, cells were washed in PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Fixation was stopped using 0.2 M glycine, pH 7.4 for 5 min, and followed by permeabilization with 0.2% saponin (Sigma), 2% FBS in PBS for 20 min. Cells were stained with anti-insulin Ab diluted at 1:50 in PBS.
with 0.1% saponin and 2% FBS for 30 min at RT followed by Alexa 594–
coupled goat anti-rabbit Abs diluted 1:200 for 30 min at RT. Stained cells were
again fixed for 10 min at RT, and the reaction stopped with 50 mM NH4Cl in
PBS for 5 min. Finally, coverslips were incubated briefly with 50 ng/ml DAPI
(Invitrogen), mounted with DABCO (Sigma), and sealed with nail polish.

Analysis of gene expression
For analysis of gene expression in vaccinia-infected cells, 5 × 10^9 HT-Kd
were infected with insulin or OVA-expressing viruses at a multiplicity of
infection of 30 and treated with drugs as described earlier. Cells transiently
were transfected with pT plasmids were rested for 16 h after transfection before simultaneous induction with 1 μg/ml Doxy and treatment with drugs for 2 (two experiments) or 5 h (one experiment). RNA was extracted from cells using Illustra RNAspin Mini kit (GE Healthcare), following the manufacturer’s instructions. One microgram RNA was transcribed to cDNA using the ImProm-II Reverse Transcription System (Promega, Charbonnieres, France) followed by primer extension PCR (qPCR) analysis. Gene
expression was performed using primers specific for activating transcription
factor 4 (ATF4), binding Ig protein (BiP), C/EBP homologous protein (CHOP),
spliced X-box binding protein 1 (XBP-1), insulin and OVA, SYBR Green Mix (Eurogentec, Angers, France), and AIB17900 equipment (Applied Biosystems). GAPDH was used as housekeeping gene for normalization,
except for spliced XBP-1 where unspliced XBP-1 was used. qPCR were performed using 2 ng/μl DNA. qPCR results are expressed as ratios between the normalized expression of test samples and that of reference samples, which are indicated in the figure legends.

Analysis of protein synthesis and degradation
To analyze general protein or insulin synthesis, or insulin degradation, we
transfected 10^7 HT cells with 24 μg plasmid DNA using 120 μg branched
polyethylenimine (Sigma-Aldrich), followed immediately by induction of protein expression by addition of 1 μg/ml Doxy and cell incubation at 37˚C for 18 h. Then 0.5 μg Epo or an equivalent volume of DMSO was added to the cells. After incubation for 2 h, the cells were detached with trypsin, washed 2× in PBS, resuspended at 5 × 10^6 cells/ml in modified RPMI 1640 labeling medium (Sigma-Aldrich) substituted with 2% FCS, glutamine, and penicillin-streptomycin (Pen/Strep) and DMSO or Epo as before, and starved for 30 min at 37˚C. After the starvation period, the cells
were then resuspended in fresh labeling medium, split into 96-well plates,
and labeled using [35S] methionine/cysteine (Expre 35S35S Protein
Labeling Mix, 259 MBq; Perkin-Elmer). When protein or insulin synthesis
was examined, labeling was followed immediately by counting of incorpo-
rated radioactivity or insulin immunoprecipitation. Cells pretreated in
the same manner were labeled as described for 15 min, followed by
resuspension in chase medium (DMEM containing 10% FCS, 100
μg/ml Pen/Strep, 100 mM PMSF, and 500 mM iodoacetamide at 4˚C for
1 h) to monitor insulin synthesis or degradation. When general protein
synthesis was examined, 5 μl of the lysate was removed at this point and added to 3 ml liquid scintillation mixture (Ultima Gold, Packard Bioscience), incubated for 1 h at RT, and counted in a Tri-Carb Liquid Scintillation Counter (Packard-Bioscience). The total or remaining
lysate was centrifuged for 5 min at 4˚C and 16,400 × g, and the supernatant was prechilled for 30 min at 4˚C using 50 μl packed Staphylococcus aureus (Sigma-Aldrich). The prechilled lysate was transferred to Protein A beads (Calbiochem) previously coated with anti- insulin Ab. After a 1-h incubation at 4˚C, the beads were washed 5× in PBS con-
taining 0.1% Nonidet-P40, dried with a syringe, and boiled in 45 μl sample buffer at 95˚C for 10 min. Finally, 15 μl of the sample was separated on a 15% SDS-PAGE and blotted on PVDF membrane. Autoradiographs taken with Fuji imaging plates and a Fuji FLA-2000 apparatus were ana-
yzed by densitometry using ImageJ.

Ag presentation assay
CD8+ T cell lines from peptide-immunized HLA-A2 transgenic HHD
mice have been described previously (19). G9C8 TCR-transgenic T cells
were obtained as frozen spleen cells and cultured in Click’s medium with
complete supplements (10% heat-inactivated FBS, 2 mM l-glutamine, 1%
Pen/Strep, 10 mM HEPES, and 50 μM 2-ME). A total of 10^6 G9C8
splenocytes were stimulated with 10^5 irradiated NOD splenocytes and 10^7
M peptide insulin-B chain 3-29 (LYVQGATLKL) (Copenhagen, Den-
mark). On days 2 and 3, 10% of T cell growth factor obtained by stimu-
lating rat splenocytes for 24 h with Con A was added. Cells were
restimulated in the same manner three times. On day 7 of the fourth
(or subsequent) stimulation(s), the G9C8 T cells were used for Ag pre-
sentation assays. For this, HT-Kd cells were transfected with 30 μg
PPI-encoding or 15 μg PI-encoding plasmids by electroporation, as
described earlier. Expression of protein was induced with 1 μg/ml Doxy
for 24 h, and cells were treated subsequently with drugs, as indicated. Where appropriate, surface peptide–MHC-I complexes were dissociated in acid (163 mM citric acid, 320 mM NaH2PO4, pH 2.6) for 1 min. Acid stripping reduced expression of H-2K^d by 94% and stimulation of G9C8 cells by transfectants by 60–90%. Acid-stripped cells were incubated with a lower (0.2 μM) Epo concentration to avoid toxicity. Cells were harvested and counted, and added at a ratio of 10.1 (10^5 HT and 10^4 T cells) to G9C8 cells in 96-well conical bottom plates for a maximum of 12 h at 37˚C and in the presence of 5 μg/ml BFA. Cells were stained with anti–CD8α,
allophycocyanin Abs diluted 1:200 for 30 min at RT, and the reaction stopped with 50 mM NH4Cl in PBS containing 1% Nonidet-P40, 200 mM PMSF, and 500 mM iodoacetamide at 4˚C for 1 h to monitor insulin synthesis or degradation. When general protein
synthesis was examined, 5 μl of the lysate was removed at this point and added to 3 ml liquid scintillation mixture (Ultima Gold, Packard Bioscience), incubated for 1 h at RT, and counted in a Tri-Carb

Results
Effect of proteasome inhibition on presentation and expression of vaccinia virus–encoded PPI
To study Ag processing of PPI, we first used previously described (19)
cytotoxic T cell (CTL) lines produced in HLA-A2–expressing transgenic mice that recognize epitopes in the three parts of the proinsulin (PI) molecule (A and B chains, C peptide). PI expression was induced by infection of HeLa APCs with a vaccinia virus encoding PPI (Fig. 1A). Incubation of APCs with a proteasome inhibitor before virus infection reduced presentation of all virus-encoded epitopes substantially (range 40–95%), whereas presentation of the corresponding synthetic peptides was not af-
ected. This suggested that presentation of PI epitopes is proteasome

To examine a possible effect of proteasome inhibition on Ag abundance, we analyzed PPI expression in virus-infected cells. APCs were preincubated with inhibitors and washed before addi-
tion of virus to avoid possible inhibitor effects on cell infection. Presentation of an epitope encoded by a vaccinia virus–encoded minigenine was not affected by proteasome inhibitors, providing further evidence against an effect of inhibitors on infection or general protein expression (data not shown). Surprisingly, the nonreversible proteasome inhibitor Epo, when present either before

Vaccinia virus infection has strong effects on cellular metabolism that may affect Ag presentation by MHC-I molecules. We therefore established a second regulated expression system, using transient transfection of HeLa cells expressing the tetracycline regulator, which had previously been transfected with the murine H-2K^d
MHC-I molecule and in this article is designated HT-Kd cells. The
drug treatment before and postinfection, abolished PPI expression almost completely (Fig. 1B). Addition of the inhibitor after virus infection had the opposite effect and resulted in increased PI detection in immunoblots. Experiments with a second drug (lactacystin) had equivalent effects, demon-
strating reduced and increased PI detection upon proteasome in-
hibition before or after vaccinia infection, respectively (Fig. 1C). These experiments allowed for two conclusions: 1) compromised proteasome function antagonizes cellular PI accumulation, possi-
ble because of inhibition of synthesis; and 2) in cells synthesizing PPI, proteasome inhibition results in PI accumulation, suggesting a role of the proteasome in PI degradation.

Effect of proteasome inhibition and drugs inducing ER stress on tetracycline-regulated expression of PPI
Vaccinia virus infection has strong effects on cellular metabolism that may affect Ag presentation by MHC-I molecules. We therefore established a second regulated expression system, using transient transfection of HeLa cells expressing the tetracycline regulator, which had previously been transfected with the murine H-2K^d MHC-I molecule and in this article is designated HT-Kd cells. The
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and PPI variants lacking one (PPI-C20S) or two (PPI-C72S) disulphide bridges, or extended by carboxyterminal tags that introduce an N-linked glycosylation site (preproinsulin-HA [PPH], preproinsulin-SIINFEKL [PPS]), combined with disulphide bridge deletion (data not shown) or not. Transiently transfected HT-Kd cells incubated with the tetracycline analog Doxy showed insulin staining that overlapped with the ER marker KDEL for PPI, but not for the cytosolic PI form, as expected (Supplemental Fig. 1). HT-Kd cells induced 16 h after PPH transfection with Doxy expressed large amounts of the protein, whereas the protein was undetectable in uninduced cells. In contrast, Doxy-induced cells expressing PH contained hardly detectable amounts of the protein (Fig. 2B).

We then tested the effect of proteasome inhibition on Doxy-regulated expression of PPH and PH. PPH expression was reduced after preincubation with Epo and when the drug was continuously present, whereas the protein was undetectable in induced cells in the absence of Epo (Fig. 2B). This suggested that PI that is ectopically expressed in the cytosol is rapidly degraded by the proteasome. Importantly, the inhibitory effect of proteasome inhibition on PPI and PPH abundance did not extend to PH and, therefore, was related to the presence of a signal peptide mediating ER targeting.

Seeking to explain the effect of ER targeting on protein expression in the presence of proteasome inhibition, we considered the possibility that ER stress could have affected synthesis of PPH and PH differentially. The UPR is a physiologic response to high protein load in the ER that relies on three ER sensor proteins that are activated on consumption of ER chaperones by unfolded protein. We then tested the effect of proteasome inhibition on Doxy-regulated expression of PPH and PH. PPH expression was reduced after preincubation with Epo and when the drug was continuously present, whereas the protein was undetectable in induced cells in the absence of Epo (Fig. 2B). This suggested that PI that is ectopically expressed in the cytosol is rapidly degraded by the proteasome. Importantly, the inhibitory effect of proteasome inhibition on PPI and PPH abundance did not extend to PH and, therefore, was related to the presence of a signal peptide mediating ER targeting.

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proteins and that downregulate general protein synthesis, upregulate levels of proteins involved in protein folding and degradation, and trigger apoptosis if the stress cannot be resolved (22). Mild ER stress can also be triggered by proteasome inhibition through ill-defined mechanisms (23, 24). In addition, ER stress can result in rapid downregulation of the synthesis of a subset of ER-targeted proteins via inositol-requiring protein 1 (IRE-1)–mediated cleavage of the corresponding mRNAs (25), providing a potential mechanism for specific downregulation of ER-targeted, but not cytosolic Pl in the presence of proteasome inhibitors.

If reduced expression of PPH was due to activation of the UPR, then other drugs inducing this condition were expected to have a similar effect. We studied the effect of tunicamycin and brefeldin A, two drugs inducing massive ER stress by blocking N-linked glycosylation and protein export from the ER, respectively. At the same time, we tested drug effects on additional PPI/I forms devoid of the nonphysiologic HA tag or lacking two disulphide bridges. The latter experiments were designed to reveal whether protein modifications that presumably alter stability modify the effect of drugs on protein abundance. Surprisingly, and in contrast with Epo, tunicamycin had only a minor effect on PPH levels, although it abolished glycosylation of PPH, which demonstrated its pharmacological activity (Fig. 3A). In contrast, brefeldin A, which retains secretory pathway proteins such as β2-microglobulin within the cell (Fig. 3B), abolished PPH expression. Tunicamycin and Epo had the same contrasting effects on abundance of PPI, demonstrating that the artificial glycosylation site and HA tag were not responsible for the observed effects (Fig. 3C). Expression of a PPH mutant lacking two disulphide bridges was also abolished by Epo, but in this case, tunicamycin strongly reduced expression (Fig. 3D). These results allowed for several conclusions: 1) a state of compromised proteasome function reduces abundance of all ER-targeted PPI/H forms irrespective of the presence of N-glycans or disulphide bridges; 2) reduced stability of ER-targeted PPH may correlate with stronger reduction of expression in the presence of ER stress inducers; and 3) different agents known to induce ER stress have distinct effects on abundance of ER-targeted PPI/H.

To directly study activation of the UPR, we quantified cellular mRNA levels for several proteins that are upregulated in its presence including IRE-1 (also called ERN1), XBP-1, BiP, CHOP, and ATF4. The latter three proteins are all induced by the PKR-like ER kinase (PERK) branch of the UPR. As expected, tunicamycin alone, or in combination with vaccinia infection or plasmid transfection, induced BiP and CHOP mRNA levels strongly and IRE-1 (ERN1) and splicing XBP-1 mRNA levels modestly, thus activating both the IRE-1 and PERK branches of the UPR. Moreover, high-level expression of vaccinia virus-encoded PPI, but not OVA, resulted in upregulation of several UPR markers, whereas transfection of PI/PPI-encoding plasmids had a modest effect, if any (Supplemental Fig. 2). However, Epo treatment alone, or in combination with cell infection or transfection, did not result in any noticeable activation of UPR markers. Therefore, the effect of Epo on PPI/H expression is not due to activation of the IRE-1 and/or PERK branches of the UPR at the transcriptional level.

In the initial wave of the UPR, regulated IRE-1–dependent decay (RIDD) of specific ER-targeted mRNAs acts as a rapid mechanism for reducing the protein load in the ER (26). Indeed, in vaccinia virus–infected cells, Epo treatment reduced PPI mRNA levels by 90% (Fig. 4A), whereas the effect on OVA mRNA levels was modest (reduction by 40%). However, Epo treatment had no significant effect on the level of PH or PPI mRNA in plasmid-transfected cells (Fig. 4A, right panel). These results suggested that the effect of Epo on the abundance of vaccinia-expressed PPI was at least partly due to reduced protein translation, whereas the reason underlying reduced abundance of plasmid-encoded PPI/H remained unclear.

Reduced protein abundance can primarily be because of a lower rate of synthesis and/or a higher rate of degradation. We studied the rates of synthesis and degradation of the different plasmid-expressed insulin forms using immunoprecipitation of metabolically labeled insulin. Pretreatment of transfected cells with Epo, present during cell starvation or not, did not alter the rate of global protein synthesis, as reflected by label incorporation during short-term pulses (Fig. 4B). Epo pretreatment also had no significant effect on synthesis of four ER-targeted insulin forms (Fig. 4C and 4D). Synthesis of PI and PPI-C72S could not be monitored because sufficient amounts of these forms could not be immunoprecipitated. Examination of insulin degradation confirmed the anticipated effects of the different protein modifications used (Fig. 4E). Thus, removal of the insulin signal peptide resulted in an extremely short half-life, whereas addition of any C-terminal tag with an N-glycosylation site increased the half-life of ER-targeted insulin. Removal of disulphide bridges from HA-tagged insulin slightly reduced half-life, whereas the difference between the half-lives of PPH and PPS suggests a modest effect of the tag sequence independent of glycosylation (Fig. 4E). Presumably as a result of different rates of degradation and possibly also synthesis, the steady-state levels of the different insulin forms dis-
played substantial differences, with high abundance of tagged ER forms, intermediate abundance of physiologic PPI, and low abundance of PPI-C72S lacking two disulphide bridges (Fig. 4F).

**Assay measuring T cell stimulation by transfectants expressing PPI and PI**

The results described so far indicated that reduced presentation of MHC-I–restricted epitopes by vaccinia virus–infected APCs (Fig. 1A) in the presence of proteasome inhibitors most likely had been caused by inhibitor effects on PPI synthesis. To identify the proteolytic pathways involved in PI and PPI degradation for presentation by MHC-I molecules, we used experimental conditions in which cells were first allowed to synthesize the antigenic protein before inhibitors were added (Fig. 5A). This was achieved by 24-h incubation with Doxy followed by a brief acid treatment to remove cell-surface K-d-insulin B15–23 complexes. The generation
of new K\textsuperscript{d}-insulin B\textsubscript{15–23} complexes from previously synthesized PPI, or from newly synthesized PI, in the presence of protease inhibitors was then monitored.

Production of antigenic epitopes was read out using TCR-transgenic G9C8 CD\textsuperscript{8+} T cells with specificity for the H2-K\textsuperscript{d}-restricted T cell epitope insulin B\textsubscript{15–23} (27), an epitope with immunodominant status in the NOD mouse that is recognized by diabetogenic T cells found in pancreatic islet infiltrates (28).

In vitro activated and restimulated G9C8 T cells secrete IFN-\(\gamma\) upon stimulation with \(10^{-8}\) M cognate peptide and H2-K\textsuperscript{d} APCs (Fig. 5B); IFN-\(\gamma\) secretion was not detectable at \(<10^{-8}\) M peptide (data not shown). Cell-surface H2-K\textsuperscript{d}–insulin B\textsubscript{15–23} complexes had a half-life of \(\sim11.5\) h (Fig. 5C), which was compatible with the use of an IFN-\(\gamma\) secretion assay carried out in the presence of brefeldin A as readout for T cell stimulation. Moreover, acid-treated H2-K\textsuperscript{d} cells transfected with PPI (or PI, data not shown) recovered full stimulatory capacity for G9C8 T cells 5 h after removal of cell-surface pMHC complexes (Fig. 5D). Therefore, acid-stripped transfectants were incubated for a minimum of 6 h before addition of T cells in subsequent experiments.

Effect of proteasome inhibitors on presentation

We next studied the efficiency of presentation of different PPI forms, as well as the effect of protease inhibitors on their presentation. Transfectants expressing PI or PPI both stimulated G9C8 cells (Fig. 5B), but intriguingly, cells expressing cytosolic PI consistently had higher stimulatory capacity than those expressing any ER-targeted Ag form (Figs. 5B and 6A). Moreover, there were marked differences between the different ER-targeted forms: N-glycosylation reduced presentation, whereas deletion of disulphide bridges had the opposite effect (Fig. 6A). Considering the results shown in Fig. 4C–F, we concluded that protein half-life and steady-state abundance were inversely correlated with presentation efficacy.

Next, we studied the effect of proteasome inhibition on the presentation of the different Ag forms. This revealed striking and unexpected differences (Fig. 6B). Whereas presentation of cytosolic PI was inhibited by MG132, presentation of all ER-targeted forms was increased. Moreover, the magnitude of the increase correlated with protein half-life, so that presentation of PPH was tripled, whereas that of PPI-C72S, the least stable ER-targeted form, was almost unchanged (Fig. 6B). This correlation applied to ER-targeted forms carrying two different C-terminal tags (Fig. 6B and 6C). The small effect of the deletion of the C72 disulphide bridge also correlated with the slightly reduced stability of this PPH variant (Figs. 4E and 6B); although we did not measure the stability of the PPS-C20S variant, it is reasonable to assume that the smaller increase in presentation upon proteasome inhibition as compared with unmodified PPS reflected reduced stability. In conclusion, protein modifications increasing protein half-life and steady-state abundance correlated negatively with efficacy of presentation and positively with enhanced presentation in the presence of proteasome inhibitors.

Possible role of alternative pathways in presentation of PI and PPI

The enhancing effect of proteasome inhibitors on the presentation of ER-targeted PPI forms suggested that other proteases might process these Ags. AAF-CMK, which inhibits both cytosolic TPPII and lysosomal TPPI, but also the cytosolic aminopeptidases bleomycin hydrolase and puromycin-sensitive aminopeptidase (29), reduced presentation of several ER-targeted Ag forms substantially (Fig. 6C), whereas batimastat, an inhibitor of vacuolar H\textsuperscript{+}-ATPase, had a smaller inhibitory effect on presentation of several ER-targeted forms and of cytosolic PI (Fig. 6B). Considering that TPPI is known both to act as an enzyme trimming epitope precursors (30) and to generate some epitopes (reviewed in Ref. 5), we tested batubindide, a more specific TPPII inhibitor.

**FIGURE 5.** Stimulation of G9C8 T cells by transfected HT cells. (A) HT-K\textsuperscript{d} cells transfected as indicated and induced with Doxy for 24 h were treated or not for 2 h with 0.5 \(\mu\)M Epo or 2 h, acid stripped, and reincubated for 8 h with or without 0.2 \(\mu\)M Epo. Lanes are mounted from two different blots and show the PI content of equivalent cell numbers. (B) HT-K\textsuperscript{d} cells transfected with PT-PPI or PT-PI were treated with 1 \(\mu\)g/ml Doxy for 24 h and incubated with G9C8 T cells for 12 h followed by flow cytometric analysis of intracellular IFN-\(\gamma\) staining in CD8\textsuperscript{+} cells. Control: G9C8 cells alone. HT-K\textsuperscript{d} cells incubated with \(10^{-8}\) M peptide insulin B\textsubscript{15–23} were used as positive control. (C) Decay of K\textsuperscript{d}/insulin B\textsubscript{15–23} complexes. HT-K\textsuperscript{d} cells were pulsed with \(10^{-8}\) M peptide insulin B\textsubscript{15–23} and incubated for the indicated time. Then cells were added to G9C8 T cells for an additional 12 h followed by flow cytometric analysis of IFN-\(\gamma\) production. (D) Recovery of K\textsuperscript{d}/insulin B\textsubscript{15–23} complexes after acid stripping. PPI-expressing HT-K\textsuperscript{d} cells were induced for 6 h with Doxy and then acid stripped. After an additional 2- to 6-h incubation, G9C8 cells were added for a 12-h incubation in the presence of BFA followed by intracellular IFN-\(\gamma\) staining and flow cytometric analysis. Asterisk indicates cells continuously incubated with BFA after acid stripping; box indicates cells not subjected to acid stripping. One of two (C and D), four (A), or eight (B) experiments is shown.
Butabindide had no effect on presentation (data not shown), arguing against a role of TPPII in degradation of ER-targeted Ag.

If the processing pathways acting on cytosolic and ER-targeted PI differ, the peptides produced in them also might be distinct; although peptide insulin B15–23 is the optimal peptide recognized by G9C8 cells (16), N- or C-terminally extended variants could be stimulatory. We reasoned that if different peptides result from degradation of PI versus PPI, the stability of the complexes formed with H-2Kd molecules might differ. To address this, we incubated HT-Kd cells with CHX, an inhibitor of protein synthesis blocking production of new complexes, and monitored the decay of H-2Kd/insulin B 15–23 complexes using G9C8 cells. We first tested the effect of CHX on expression of H2-Kd and observed a reduction by 33% after a 4-h incubation (Fig. 7A). Under the same conditions, presentation of PPI was reduced by 30%, whereas that of PI was reduced by 75% (Fig. 7B). Thus, pMHC complexes formed upon degradation of PPI were significantly more stable than those formed upon degradation of PI, consistent with production of distinct peptides from the two Ag forms.

**Discussion**

We have demonstrated that ER targeting of PI affects three parameters: protein abundance in the presence of proteasome inhibition, efficacy of presentation, and effect of proteasome inhibition on Ag presentation. Moreover, in the case of ER-targeted Ag forms, we find that the two latter parameters correlate with modifications altering protein half-life. Finally, we report evidence suggesting that, for a given Ag, subcellular localization may affect the exact nature of an epitope presented. Collectively, these results highlight the important role of subcellular Ag localization in MHC-I Ag processing.

Searching for a mechanism explaining reduced PI expression in the presence of proteasome inhibitors, the fact that the phenomenon was limited to ER-targeted PI forms led us to speculate that ER stress was involved. The UPR has a physiologic and dual role in production of insulin by β cells: functional PERK and IRE-1 branches of the UPR are required for β cell survival and function; however, chronic activation of IRE-1 can compromise insulin production (31, 32). Both IRE-1–dependent cleavage of PPI mRNA and global attenuation of protein translation mediated by the PERK-dependent pathway were candidate mechanisms explaining reduced PPI synthesis in the presence of proteasome inhibitors (22). However, we did not find evidence for significant IRE-1–dependent XBP-1 splicing or for PERK-dependent upregulation of BiP, CHOP, or ATF4 in Epo-treated cells. Moreover, the fact that tunicamycin, which caused significant activation of both the IRE-1 and the PERK-dependent pathways, did not reduce PPI levels argued against involvement of these pathways. Nevertheless, the only significant effect of Epo treatment was the reduction of PPI mRNA levels by 90% in vaccine-infected cells. PPI mRNA can become a substrate of the RIDD pathway upon chronic IRE-1 hyperactivation or when IRE-1 is overexpressed (32, 33). Given that this pathway involves the nuclease activity of activated IRE-1, which is also responsible for the cleavage of XBP-1 mRNA, absence of spliced XBP-1 mRNA in Epo-treated cells at first sight argues against an involvement of RIDD. However, dissociation between XBP-1 splicing and IRE-1–dependent decay of other mRNAs, which seems to require ill-defined other
components of the UPR, has recently been described (26). Reduced PPI mRNA levels are likely to result in reduced translation and, therefore, in low levels of PPI in vaccinia virus–infected cells. In contrast, our experiments do not provide a mechanistic explanation for the reduced abundance of ER-targeted insulin in transfected HT-Kd cells. In these cells, Epo treatment altered neither the rate of global protein synthesis nor the rate of synthesis of ER-targeted insulin forms. Therefore, proteasome inhibition may lead to increased degradation of ER-luminal insulin, but the mechanism remains unclear (see also later). Whatever the precise mechanism of stress-induced suppression of PPI/H expression, our results suggest that caution should be applied when interpreting the effect of proteasome inhibition on MHC-I–restricted Ag presentation, especially in studies concerning proteins of the secretory pathway.

Inhibition of PPI synthesis upon proteasome inhibition has previously been observed in glucose-stimulated primary and immortalized murine β cells (34). Proteasome inhibition in β cells also stimulated insulin release, presumably to reduce the protein load in the ER. Considering that a similar mechanism might explain increased presentation of epitope insulin B15–23 by Epo-incubated cells, we have performed mixing experiments in which HT-Kd cells transfected with PPI, themselves unable to present the Kd-restricted epitope, were mixed with untransfected HT-Kd APCs. However, no evidence for release of antigenic material was observed in these experiments (data not shown).

The fact that ectopic cytotoxic expression of an ER protein increases its MHC-I presentation is most likely due to the rapid degradation of cytotoxic PI, similar to a previous case reported by Eisenlohr’s group (12). However, although their study did not find an effect of Ag modifications such as removal of disulphide bridges or of an N-glycosylation site and efficacy of presentation, we demonstrate a correlation between these parameters. This suggests a link between ER quality control and MHC-I Ag processing in the case of PI, consistent with previous studies in which altered expression of ER folding chaperones modulated presentation efficiency (11, 35).

The mechanism that underlies the relation between Ag half-life, on the one hand, and the quality and quantity of the effect of proteasome inhibition on presentation, on the other hand, remains difficult to ascertain. Indirect effects, for example, effects mediated by a proteasome requirement for specific elements of one of the different ERAD pathways (10), cannot be ruled out. However, simpler scenarios are conceivable in which both inhibition and enhancement of Ag presentation by proteasome inhibitors can be explained by direct effects on Ag cleavage by the proteasome. It is well documented that the proteasome, like other proteases, can both destroy and produce antigenic peptides (36). In this case, PI derived from stable ER-targeted Ag forms may encounter the proteasome in a different physical state than PI derived from less stable ER-targeted or unstable cytotoxic PI. We have performed in vitro digestion experiments with purified proteasome and oxidized or reduced and carboxymethylated proinsulin that have shown that oxidized PI is cleaved exclusively after Cys19, whereas cleavages flanking the epitope are observed for reduced PI (H.-T. Hsu and P. van Endert, unpublished observations). Stable ER-targeted PI forms will be oxidized in the ER and may encounter the proteasome in an oxidized form, which might favor epitope destruction by dominant cleavage after Cys19. In contrast, PI remaining in the cytosol, a reducing environment, may undergo a sufficient number of cleavages flanking the epitope to shift the net proteasome effect to epitope generation rather than destruction. Alternatively, it is possible that ER-targeted and cytotoxic PI forms have access to different proteasome forms with distinct cleavage specificities, as previously discussed by Golovina and associates (37).

More complex pathways may also underlie the observations described in this article. Comprehensive investigations of larger epitope panels suggest that the standard model of endogenous Ag processing for MHC-I molecules may be inappropriate reductionist in the case of many epitopes, and that Ag processing may involve additional compartments and intracellular transport events (38). For example, the results obtained with AAF-CMK, buta-binderide, and bafilomycin in this study suggest the implication of a lysosomal protease. Although we have not been able to obtain consistent results with pharmacological induction and inhibition of macroautophagy (data not shown), a role for this pathway in the presentation of ER-targeted PPI also cannot be ruled out. Two mechanisms that might be involved in such a pathway, donation of autophagosomes membranes from ER-like compartments, and engulfment of ER membranes by autophagosomes, have been described previously (39, 40). A pathway in which Ags escape from autophagosomes into the cytosol where they tend to be degraded by the proteasome, similar to that described by English et al. (41) for an HSV protein, might also handle ER-targeted PI.

In summary, we report that ER targeting can have a profound impact on the processing of an MHC-I–restricted epitope. This observation first underlines the importance of monitoring subcellular Ag targeting and Ag expression in the presence of proteasome inhibitors in Ag processing studies. Furthermore, our results highlight the complexity of Ag processing pathways, which remains to be fully characterized. Lastly, our findings may be of importance in autoimmune T1D. It has been shown using mice expressing B7 in β cells that triggering of T1D by vaccination with insulin-expressing plasmids requires ER targeting of insulin (42).

Disclosures
The authors have no financial conflicts of interest.

References


**Supplementary Figure 1.**

PI- or PPI-transfected HT cells induced with 1 µg/ml Doxy for 24h were stained for insulin (red) and the endoplasmic reticulum marker KDEL (green) with nuclear counterstaining by DAPI (blue).
Supplementary Figure 2. Effect of drugs on expression of ER stress markers. In A, HeLa cells were treated for 2h with solvent, 0.5 μM Epo, or 5 μg/ml tunicamycin; then cells were either infected with PPI- or OVA-encoding vaccinia viruses for 4 h, or incubated for 4 h in medium, before harvesting, RNA extraction, and examination by quantitative PCR. All values obtained were normalized with respect to the house-keeping gene GAPDH. In A, the left hand panel shows the ratio between gene expression by untreated cells and expression by cells treated with drugs or infected. The right hand panel shows the ratio between cells infected only and cells infected and treated with drugs. B shows the results of an equivalent experiment in which HT cells were only transfected or drug-treated (left hand panel), or transfected and treated with drugs (right hand panel). Ratios in B were computed like in A.