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Ralf M. Leonhardt, Dorothee Fiegl, Elke Rufer, Axel Karger, Barbara Bettin and Michael R. Knittler

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Post-Endoplasmic Reticulum Rescue of Unstable MHC Class I Requires Proprotein Convertase PC7

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The function of the peptide-loading complex (PLC) is to facilitate loading of MHC class I (MHC I) molecules with antigenic peptides in the endoplasmic reticulum and to drive the selection of these ligands toward a set of high-affinity binders. When the PLC fails to perform properly, as frequently observed in virus-infected or tumor cells, structurally unstable MHC I peptide complexes are generated, which are prone to disintegrate instead of presenting Ags to cytotoxic T cells. In this study we show that a second quality control checkpoint dependent on the serine protease proprotein convertase 7 (PC7) can rescue unstable MHC I, whereas the related convertase furin is completely dispensable. Cells with a malfunctioning PLC and silenced for PC7 have substantially reduced MHC I surface levels caused by high instability and significantly delayed surface accumulation of these molecules. Instead of acquiring stability along the secretory route, MHC I appears to get largely routed to lysosomes for degradation in these cells. Moreover, mass spectrometry analysis provides evidence that lack of PLC quality control and/or loss of PC7 expression alters the MHC I-presented peptide profile. Finally, using exogenously applied peptide precursors, we show that liberation of MHC I epitopes may directly require PC7. We demonstrate for the first time an important function for PC7 in MHC I-mediated Ag presentation. The Journal of Immunology, 2010, 184: 2985-2998.

Antigenic peptides are loaded onto MHC class I (MHC I) molecules in the endoplasmic reticulum (ER) within a large multisubunit complex known as the peptide-loading complex (PLC) (1). One major function of this complex is to optimize the peptide cargo transferred into the binding groove of MHC I in a sense that only stably binding, high-affinity ligands are loaded (1). Consequently, cells that express a defective PLC, owing to absence or mutation of its central constituents’ TAP (2, 3) or tapasin (4), generate a large fraction of structurally fragile MHC I-peptide complexes that display low thermal stability and a high tendency to disintegrate during transport along the secretory route or at the cell surface. Upon loss of their antigenic ligand, empty MHC I molecules are not only useless for immunosurveillance by cytotoxic T cells, but may become harmful when they indiscriminately pick up peptides from the extracellular milieu.

We recently showed that reconstitution of the TAP-deficient cell line T2, with the truncated transporter 1-2ΔN, fully restores peptide transport and supply to MHC I, but fails to restore the quality control function of the PLC (3). In the mutant PLCs of these cells, MHC I largely acquires suboptimal ligands, which fail to confer normal stability. We note that this strongly resembles the effects of viral evasion targeting the function of the loading complex (5-7). Following ER-export, however, initially labile MHC I molecules with low structural integrity were found to substantially improve over time, with their stability eventually arriving at the cell surface as highly stable molecules (3). Similar observations have also been described for tapasin-deficient cells (4), suggesting that post-ER stabilization of MHC I-peptide complexes is likely to become generally applied under circumstances in which unstable MHC I peptide complexes leave the ER and enter the exocytic pathway. The underlying mechanism was shown to require a class of serine proteases, the so-called proprotein convertases (pPCs) (3), and is likely to involve peptide exchange in late secretory compartments. Consistent with this finding, others have found pPCs to generate MHC I-ligands in the trans-Golgi network (TGN) of TAP-deficient cells (8, 9).

The human genome encodes at least seven highly related members of the subtilisin/kexin-like pPC family, three of which display a highly restricted expression pattern in testis (PC4) or endocrine/nerve/endoctrine cells (PC1 and PC2) (10). The remaining four members (furin, PACE4, PC5, and PC7) show a widespread tissue distribution and primarily reside in the TGN, in post-TGN vesicles or in the endocytic system (10), where they process a large number of polypeptides of cellular (11) and/or pathogenic (12) origin. In particular, viral fusion proteins frequently require cleavage-activation by pPCs to become functional (13), so that pPC-dependent processing might introduce important viral epitopes to the MHC I Ag presentation pathway. However, which pPCs are involved in generating and processing MHC I ligands in the compartments of the late secretory route is unknown.

In this study we found that, of the four broadly expressed pPCs, only PC7 and furin are transcribed in T2 cells. To investigate whether furin, PC7, or both are involved in post-ER stabilization of MHC I complexes, we performed specific and constitutive silencing (RNA interference) of the two pPCs in the cell line T2(1-2ΔN), characterized...
by a defective quality control in the PLC (3, 14). Our experiments show that in the absence of PC7, unstable MHC I molecules are not rescued in post-ER compartments during their intracellular transport to the cell surface. Consistent with this finding, PC7-silenced T2(1-2ΔN) transfectants [T2(1-2ΔN/PC7)] are characterized by delayed, reduced, and unstable surface expression of MHC I complexes. In contrast, no such effects on intracellular MHC I maturation and surface expression could be observed for T2(1-2ΔN) cells knocked down for furin [T2(1-2ΔN/FUR)]. Moreover, in T2(1-2ΔN/PC7)] cells a substantial amount of post-ER-MHC I routes to the lysosomal pathway. In support of our findings, MALDI-mass spectrometry (MS)-analysis provides experimental evidence that the MHC I-presented peptide profile is altered in the absence of PLC quality control and/or lack of PC7 function. Furthermore, using exogenously applied peptide precursors, we show that liberation of MHC I epitopes may directly require PC7. Based on our findings, we suggest that the post-ER stabilization of suboptimally loaded MHC I molecules occurs in the TGN and/or post-TGN vesicles via an exchange of bound peptide ligands for ones that depend on the proteolytic activity of the pPC7 for their generation.

Materials and Methods

Cell lines and cell culture

T2 is a human TAP-deficient lymphoblastoid cell line expressing HLA-A2 and HLA-B51 (15). Transfectants of T2 expressing rat TAPwt or TAP1-2ΔN were cultured in IMDM (Invitrogen, Carlsbad, CA) supplemented with 1 mg/ml G418 (PAA) and/or 750 ng/ml puromycin (Sigma-Aldrich, St. Louis, MO) (16). The human hepatoma cell line HepG2 (17) was cultured in DMEM (Invitrogen) supplemented with 10% FCS, 1 mM sodium pyruvate (Invitrogen), 2 mM l-glutamine (Invitrogen) and non-essential amino acids (Invitrogen). All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

RNA interference

Vectors encoding short hairpin RNA (shRNA) targeting furin (5'-GGGCCTTCATGACACCTCATT-3') or PC7 (5'-GCTATGCACCTCACTTAAA-3') were constructed on the basis of the plasmid pSUPER (Oligoengine, Seattle, WA) according to the manufacturer's instructions. Constructs were sequenced before cotransfection with pPUR (Promega, Madison, WI) into G418-resistant T2(1-2ΔN) cells by electroporation using a Bio-Rad gene pulser (Bio-Rad, Hercules, CA) at 270 V and 500 μF. After cotransfection with puromycin (750 ng/ml) and G418 (1 mg/ml) for 4-6 wk, stable transfectants were subcloned and screened for the silencing of furin and PC7 by RT-PCR and Western blot analysis. For small interfering RNA (siRNA)-mediated knockdown of PC7 annealed double-stranded HP (HiPerformance) validated with 1 mg/ml G418 (PAA) and/or 750 ng/ml puromycin (Sigma-Aldrich, Cambridge, U.K.). The rabbit polyclonal antiserum to human tapasin (Abcam, Cambridge, U.K.) was used as control. FITC-, tetramethylrhodamine isothiocyanate (TRITC-) and HRP-conjugated Abs were purchased from Dianova (Hamburg, Germany).

Immunofluorescence

T2 cells were fixed at room temperature for 15 min in 3% paraformaldehyde, quenched with 10 mM glycine for 10 min, permeabilized with 0.1% saponin (Sigma-Aldrich) and incubated serially with the indicated primary and appropriate fluorescence-labeled secondary Abs. All images were captured by using an Axiovert 200M/Amotape microscope (Zeiss, Goettingen, Germany). Colocalization of fluorophores was measured using the Axiovision colocalization software module (Zeiss). The software automatically analyzes the number of pixels by which two different fluorophores are colocalized.

Preparation of endomembranes from T2 transfectants

Five × 10⁶ cells were incubated in ice-cold 10 mM Tris pH 7.5 for 10 min. Next, the cells were homogenized and the nuclei were removed by centrifugation at 1,600 × g; 20 min HEPES pH 7.5 / 25 mM CH₃COOK / 5 mM (CH₃COO)₂Mg4H₂O / 1 mM DTT / Complete protease inhibitor-mixture (Roche, Basel, Switzerland) were added to the supernatant and centrifuged at 1,600 × g. Afterward, the postnuclear supernatants were subjected to ultracentrifugation at 100,000 × g to isolate endomembranes.

Pulse-chase analysis, immunoprecipitation, endoglycosidase H digestion, and Western blotting

Cells were starved for 2 h in methionine/cysteine-free RPMI 500 μCi Promix (Amersham) was added for 30 or 60 min. The chase was initiated by the addition of a 10-fold excess of unlabeled methionine/cysteine. Equal numbers of cells were lysed in PBS containing 1% Triton X-100 (Sigma-Aldrich). Immunoprecipitations were performed from equivalent amounts of precleared lysates by using anti-MHC I Abs. For identification of immunoprecipitated MHC I, precipitated complexes were separated by SDS-PAGE. Fluorographs were scanned by a Chromoscan 3 microdensitometer (Lobei, Gateshead, U.K.) or quantified using GellEval 1.22 software (FrogDance Software, Dundee, U.K.). Immunoprecipitation, endoglycosidase H (Endo H) digestion, and Western blotting were performed as described earlier (16).

Flow cytometry

Flow cytometry was performed as described previously (16). To acid-remove surface MHC I molecules, cells were treated with IMDM/50 mM Na-citrate pH 3.0 for 3 min before neutralization with 150 mM NaH₂PO₄ pH 10.5. Treatments with hexa-n-arginine (HNA) (Calbiochem, San Diego, CA) at 100 μM were performed for 14 h at 37°C in FCS-free IMDM after an acid-wash. Decay of surface-MHC I was assessed in standard brefeldin A (BFA) assays, in which cells were treated for different times with 10 μg/ml of the drug. MHC I surface levels were determined with Ab 4E. For thermostability assays with living cells, T2 and 721.220 transfectants were heat shocked at 45–60°C for 10 min. To examine the stability of surface-HLA-B51, cells were directly immunostained with anti-MHC I Ab 4E. To analyze the stability of intracellular HLA-B51, cells were first fixed, permeabilized, and stained with Ab 4E. The specificity of intracellular MHC I staining was controlled with different human cell lines not expressing HLA-B51 (data not shown). To characterize endosomal recycling of MHC I priming (Sigma-Aldrich) was applied for 4 h at a concentration of 250 μM.

MS analysis of HLA-I associated peptides

HLA-B51-associated self-peptides were analyzed by MALDI-MS. For the isolation of surface-MHC I molecules, cells were preincubated in the cold.
for 2 h with 4E Ab. Afterward, unbound anti-HLA-B51 Abs were removed by extensive washing, and cells were lysed in 1% n-octylglucoside for 30 min. Because T2 cells do not synthesize detectable amounts of immunoglobulins by themselves (data not shown), postnuclear lysates could be directly incubated for 2 h with protein A-Sepharose. The successful isolation of surface HLA-B51 was controlled by Western blot, and the obtained MHC I signals were quantified by densitometric scanning of four different blot exposures. Beads with immunoisolated MHC I molecules were transferred into an ultrafiltration tube with a 3-kDa cutoff (Millipore, Bedford, MA), washed with 10 volumes of 20 mM Tris, pH 7.8, 0.1% Zwittergent 3-12 (Calbiochem, San Diego, CA) and 20 volumes of bidistilled water. Peptides were eluted by incubation in 0.1% trifluoroacetic acid in bidistilled water for 30 min, followed by ultrafiltration and lyophilization. MALDI-MS analysis of the eluted peptides was performed on a Bruker Ultraflex Instrument and spectra were processed by flexAnalysis 2.0 software (Bruker Daltonics, Bremen, Germany). Obtained data were normalized to the relative amount of immunoisolated HLA-B51 molecules determined by immunoblot.

Results

The expression profile of pPCs in human T2 cells is limited to PC7 and furin

We previously observed that reconstitution of the human TAP-deficient cell line T2 with the truncated peptide transporter 1-2ΔN causes the formation of unstable MHC I molecules, which after their export from the ER undergo stabilization by a mechanism depending on pPCs (3). To investigate which members of the pPC family (10) are necessary to allow this post-ER rescue to occur, we first determined by RT-PCR the T2-specific expression profile of the four broadly expressed pPCs (furin, PC5, PC7, and PACE4).

As shown in Fig. 1A, transcripts for PC7 and furin were detected in T2 cells, whereas PC5 and PACE4 were completely absent. In contrast, the human hepatoma cell line HepG2, which served as a positive control in this experiment, expressed all four pPCs. To confirm the expression of PC7 and furin in T2 cells also on the protein level, we performed Western blot analysis, which demonstrated the presence of both pPCs in a T2-derived endomembrane fraction (Fig. 1B). Moreover, the cellular t1/2 of both pPCs in T2 cells was similar and was determined to lie in the range of ~2 h (Fig. 1C). As expected, furin was predominantly detected in compartments that also contained the TGN marker TGN46, whereas the cellular distribution of PC7 displayed only a minor overlap with that of furin and TGN46 in most T2 cells (Fig. 1D, compare first, second and third rows). In fact, comparative immunofluorescence analysis of intact and permeabilized cells demonstrated that in the T2 cells PC7 mostly localizes to vesicles that line up immediately beneath the plasma membrane and apparently not on the plasma membrane itself (Fig. 1D, fourth row). Thus, consistent with the work of Wouters et al. (23), PC7 and furin appear to be spatially separated to a large extent in the cell.
In summary, according to the above pPC expression profile, PC7 and furin are the only convertases in T2 cells that might potentially be involved in post-ER stabilization of MHC I.

PC7 but not furin is required for normal MHC I surface levels in the context of a defective PLC

PC7 or furin (or both) may be responsible for the post-ER rescue of unstable MHC I formed in the T2-derived transfectant T2(1-2ΔN) that expresses a defective PLC. To distinguish between possible roles for PC7 and furin, we stably silenced either of the two pPCs in the T2(1-2ΔN) cell line via specific shRNA expression.

RT-PCR analysis showed that the knockdown of either pPC in the stable cell lines T2(1-2ΔN/FUR↓) and T2(1-2ΔN/PC7↓) was efficient and specific (Fig. 2A, upper panel). To quantify the silencing efficiency we performed quantitative PCR confirming 8–10-fold lower mRNA levels for furin and PC7, respectively (Fig. 2A, lower panel). To further demonstrate specificity of pPC silencing, we performed Western blotting using detergent-solubilized endo-membrane fractions of the different transfectants (Fig. 2B). As expected from the experiments in T2 cells (Fig. 1B), the translation products of the two pPCs were readily detected in the original cell line T2(1-2ΔN), whereas they were almost undetectable in their respective knockdown derivatives. Quantification of the obtained

FIGURE 2. PC7 but not furin is required for high MHC I surface levels in T2(1-2ΔN) cells. A, pPC or GAPDH expression in T2(1-2ΔN) or its stable knockdown derivatives T2(1-2ΔN/FUR↓) and T2(1-2ΔN/PC7↓) was analyzed by RT-PCR (upper panel) or qPCR (lower panel). B, Cell lysates and membrane extracts of the different T2 transfectants were analyzed by Western blot using the indicated Abs. C, Surface expression of HLA-B51 was assessed by flow cytometry using the Ab 4E (filled histograms, left panel). In additional experiments, mAb against transferrin receptor (CD71) was used as control (filled histograms, right panel). Background staining was analyzed by incubating with secondary Ab alone (unfilled histograms, left and right panels). One representative experiment of two independent experiments is shown. D, HDA sensitivity of MHC I surface expression in T2(1-2ΔN) cells silenced for furin or PC7. Cells were acid washed to remove assembled surface MHC I and incubated for 14 h in the presence of 100 μM pPC-inhibitor HDA. Subsequently, cells were immunostained with the mAb 4E to assess surface expression of HLA-B51 by flow cytometry (left panel). Surface MHC I staining in the presence of HDA is plotted as a percentage of reduction in MFI compared with the MFI of the control experiments (right panel). One representative result of three separate experiments is shown.
signals by densitometric scanning revealed a reduction for furin and PC7 by >98% compared with the original cell line T2(1-2ΔN), whereas the protein levels for TAP1, TAP2 (or its truncated variant 2ΔN) and ER60 were not affected by the silencing vectors.

Next, to assess whether either of the two pPCs has a role in Ag presentation, we determined the steady-state surface levels of the MHC I allele HLA-B51 in the different T2 transfectants by flow cytometry (Fig. 2C, left panel). As expected from our previous study, T2 cells transfected with wild type (wt; TAPwt) or truncated (1-2ΔN) TAP had similar MHC I surface levels, in the latter case allowed for by a functional pPC-dependent post-ER stabilization mechanism (3). As shown in Fig. 2C, additional furin knockdown in T2(1-2ΔN) did not alter MHC I expression, suggesting that furin is unlikely to be necessary for the proper operation of this mechanism. In striking contrast, however, PC7 knockdown caused a substantial reduction in the HLA-B51 steady-state surface levels in the context of 1-2ΔN-expression, (Fig. 2C, left panel). This reduction was specific, because transferrin receptor was expressed equally on all cell lines (Fig. 2C, right panel), indicating an essential role for PC7 in the post-ER rescue of unstable MHC I molecules. Moreover, we note that we also observed substantial downmodulation of HLA-B51 surface expression in T2(1-2ΔN) cells via transient transfection with either of three unrelated PC7-targeting siRNAs (data not shown), further underscoring the importance of and specific requirement for PC7 in the rescue of unstable HLA-B51.

To further corroborate this finding, we pharmacologically inhibited pPCs in all above cell lines using the cell-permeable peptide HDA, which efficiently blocks both furin and PC7 with an inhibition constant (Kᵢ) of 106 and 1875 nM, respectively (24). In our previous studies we observed that HDA treatment specifically impaired MHC I surface expression in T2(1-2ΔN), but not in T2 (TAPwt) cells, consistent with a requirement for post-ER stabilization of MHC I only in the former cell line (Fig. 2D) (3). In keeping with our observation that furin is not required for this process to occur, the furin-silenced cell line T2(1-2ΔN/FUR) fully retained the sensitivity to the drug observed in the mother cell line. However, HDA did not further affect (the already at steady-state low) MHC I surface levels on T2(1-2ΔN/PC7) cells, suggesting that it is indeed the target of the drug that is downregulated in this cell line (Fig. 2D). We additionally note that, because HDA has no effect on HLA-B51 levels in T2(TAPwt) cells (Fig. 2D) (3), PC7 is unlikely to have a general effect on loading or trafficking of MHC I in T2-derived cell lines, but is only specifically required in cells that fail to generate stable MHC I in the ER. We provide both genetic and pharmaceutical evidence that PC7 is required, whereas furin is dispensable for efficient post-ER rescue of unstable MHC I.

**Stability of surface MHC I is severely compromised in T2(1-2ΔN) transfectants lacking PC7**

Our previous studies (3) demonstrated that the MHC I molecules that formed in the cell line T2(1-2ΔN) were initially unstable, but gained stability after export from the ER to finally accumulate at the cell surface as highly stable molecules. Hence, loss of surface MHC I in the PC7-silenced cells would likely be the consequence of a deficient stabilization process normally operating along the secretory route.

To address this directly, we performed thermostability experiments that make use of the established correlation between heat resistance of MHC I and the affinity of its peptide cargo (3, 4). To this end, lysates of the different T2 transfectants were incubated for 1 h at 4°C or 37°C before intact assembled MHC I molecules were immuno-isolated with the HLA-B–specific conformation-sensitive mAb 4E (21). In accordance with our earlier findings (3), HLA-B51 was largely heat-stable in TAPwt transfectants (Fig. 3A, lane 1; for quantification, see Fig. 3B), whereas in cells expressing truncated TAP (1-2ΔN), ≥50% of the MHC I peptide complexes did not survive the incubation at 37°C (Fig. 3A, lane 2; for quantification, see Fig. 3B). Thermal instability of HLA-B51 was also seen for the T2(1-2ΔN) transfectants silenced for furin or PC7 (Fig. 3A, lanes 3 and 4; for quantification, see Fig. 3B). To elucidate whether the observed heat sensitivity of MHC I was due to suboptimal peptide binding, lysates were preincubated with a high affinity HLA-B51 peptide ligand (QPRAPIPRP) before incubation at 37°C. As can be seen from Fig. 3A (for quantification, see Fig. 3B), the pretreatment with peptides lead to a significant stabilization of HLA-B51 in T2 (1-2ΔN), T2(1-2ΔN/FUR) and T2(1-2ΔN/PC7). Thus, all T2-transfectants expressing the TAP variant 1-2ΔN have a comparable defect in the intracellular formation of stable MHC I peptide complexes.

We next compared the thermostability of intracellular to that of cell-surface HLA-B51 complexes in intact cells. Because assemblagem HC I molecules have a much higher heat resistance under physiologic conditions than in detergent extracts (3, 25), transfectants were heat-shocked for 10 min at 60°C to disrupt unstable MHC-peptide complexes or were left untreated. To determine the thermostability of intracellular HLA-B51, cells were first fixed, permeabilized, and immunostained with the conformation-sensitive Ab 4E (Fig. 3C, left column), whereas cells were directly immunostained (Fig. 3C, right column) for flow cytometry for the analysis of surface HLA-B51.

As expected from our previous studies (3) T2(TAPwt) is characterized by a high thermostability of intracellular HLA-B51 at 60°C, whereas MHC I complexes in T2(1-2ΔN), T2(1-2ΔN/FUR) and T2(1-2ΔN/PC7) disintegrated to a drastic extent under these conditions (60–70% reduction of the intracellular immunostaining). However, despite the abundant population of largely fragile intracellular MHC I molecules, we found that T2(1-2ΔN) harbors highly stable surface-HLA-B51, in fact similar in stability to that found for T2(TAPwt) (Fig. 3C, right column) (3). A comparable high thermostability of surface-HLA-B51 was also observed for the furin-silenced cell line T2(1-2ΔN/FUR). However, most interestingly, surface MHC I in T2(1-2ΔN/PC7) cells readily disintegrated in response to the heat shock, indicating that PC7 is essential to allow the process of post-ER stabilization to occur. To control the specificity of our observation for T2(1-2ΔN/PC7) cells, we performed a second independent PC7 knockdown experiment with T2(1-2ΔN) cells in which we used siRNA oligonucleotides targeting a sequence of the PC7 gene different from that of the PC7-shRNA construct (Fig. 3D; Materials and Methods). The effective and specific knockdown of PC7 by siRNA was verified by Western blot experiments with lysates from T2(1-2ΔN) cells that were transfected or not with PC7 or control siRNA (Fig. 3D, left panel). In agreement with the results shown in Fig. 3C, we observed that PC7 siRNA silenced T2(1-2ΔN) cells are characterized by thermolability of intracellular and surface-expressed HLA-B51, whereas in contrast the control siRNA transfectants possess the same high thermostability of surface MHC I as the nontransfected T2(1-2ΔN) cells (Fig. 3D, middle and right panels).

Interestingly, improved surface-over-intracellular stability of HLA-molecules was also observed in LCL 721.220 cells expressing tapasin-C95A (data not shown), suggesting that post-ER rescue of fragile MHC-peptide complexes occurs independently of what particular molecular mechanism actually generates these unstable MHC I molecules. Consistent with the concept of PC7 being responsible for this phenomenon, we found PC7 to be expressed in LCL 721.220 cells by RT-PCR (data not shown). Importantly, we note that this finding is consistent with the results of
Figure 3. Thermostability of MHC I molecules in T2(1-2ΔN) cells silenced for furin or PC7. A, Lysates of T2 transfectants were pretreated or not with 10 μM HLA-B51-binding peptide (QPRAPIRPI) and incubated for 1 h at 4°C or 37°C. Thermostability of HLA-B51 was analyzed by immunoprecipitation of assembled MHC I using the conformation-sensitive Ab 4E. Precipitates were analyzed in Western blots probed with the Ab 3B10.7. B, The levels of precipitated MHC I H chain were quantitated from the Western blot by densitometric scanning and peak integrals were plotted as bar graphs in arbitrary units. C, Thermostability of intracellular and cell surface MHC I. Transfectants were collected in PBS/0.1% NaN₃ and heat shocked at 60°C for 10 min. Assembled intracellular (left column) or surface (right column) HLA-B51 was immunostained with the anti-MHC I Ab 4E and analyzed by flow cytometry. Background staining was analyzed by incubating with secondary Ab alone (unfilled histograms). Data are representative of two independent experiments. D, Effect of siRNA mediated silencing of PC7 on the thermostability of intracellular and surface MHC I of T2(1-2ΔN) cells. To knock down PC7 expression, siRNA specific for PC7 were transfected into T2(1-2ΔN). As a negative control, AllStars Negative Control siRNA (Qiagen) was used. To verify the knockdown of PC7 cell lysates of T2(1-2ΔN) and the siRNA transfectants were analyzed by Western blot using the indicated Abs (left panel). Cells from the same knockdown experiment were collected in PBS/0.1% NaN₃ and heat shocked for 10 min. Assembled intracellular (middle panel, left column) or surface (middle panel, right column) HLA-B51 was immunostained with the anti-MHC I Ab 4E and analyzed by flow cytometry (middle panel). Data are representative of three independent experiments (see histogram, right panel).
Williams et al. (4), who demonstrated HLA-B*2705 expressed at the cell surface of tapasin-deficient LCL 721.220 cells to be vastly more stable than its respective intracellular fraction, whereas when tapasin was coexpressed, intracellular HLA-B*2705 was highly stable. Altogether, this strongly suggests that rescue of suboptimally loaded HLA-molecules cannot only occur in different types of cells and in the context of different alterations to the PLC; it also can involve different HLA-alleles (e.g., HLA-B*5101, HLA-B*2705).

Combined with our data on steady-state surface-MHC I levels and HDA sensitivity, this finding strongly suggests that PC7 is the pPC required for the rescue of unstable MHC I molecules generated in the context of a PLC devoid of a proper quality control function.

Newly synthesized MHC I is largely routed to lysosomes for degradation in T2(1-2ΔN/PC7↓) cells

To analyze the maturation and stabilization of MHC I complexes directly during their intracellular transport to the surface, we performed comparative pulse-chase experiments with the different T2 transfectants (Fig. 4). To this end, cell extracts from different time points after pulse-labeling were heat-treated at 37°C for 1 h in the presence or absence of MHC I-binding peptides. Thermoresistant MHC I was subsequently immuno-isolated with conformation-sensitive mAb 4E and incubated with Endo H. This experimental setup allows us to gain insight into how the thermostability of the pulse-labeled post-ER MHC I population develops over time. In accordance with our earlier observations (3), MHC I molecules in T2(TAPwt) cells had obtained full thermostability at the time when they were exported from the ER. In contrast, in the cell lines T2(1-2ΔN) and T2(1-2ΔN/FUR↓), the ER-exported MHC I was initially unstable, but then steadily enhanced its thermostability during post-ER transport to the cell surface (Fig. 4A–C, compare black versus gray bars). In contrast, in the case of T2(1-2ΔN/PC7↓), post-ER MHC I molecules do not display such intracellular improvement of stability (Fig. 4D, compare black versus gray bars), but seem to have an accelerated protein turnover. In support of this, pulse-chase experiments that were performed in the presence of the lysosomotropic reagent chloroquine and stabilizing peptides revealed that in T2(TAPwt), T2(1-2ΔN), and T2(1-2ΔN/FUR↓) only a minor fraction of MHC I molecules enters the chloroquine-sensitive pathway, whereas in T2(1-2ΔN/PC7↓) a substantial amount of post-ER MHC I routes to lysosomal degradation (Fig. 4D, compare gray versus white bars).
The kinetics of HLA-B51 maturation in the respective T2 derivatives provide evidence that a PC7-dependent process rescues unstable MHC I from lysosomal degradation and instead allows this population to acquire optimal high affinity peptide ligands in a post-ER compartment.

**Surface HLA-B51 in T2(1-2ΔN/PC7↓) cells presents an altered peptide repertoire**

Our experiments in Figs. 3 and 4 show that, in T2(1-2ΔN/PC7↓) cells, intracellular and surface MHC I are characterized by remarkable physical lability and increased susceptibility to lysosomal degradation. Thus, a higher turnover rate of surface-HLA-B51 in T2(1-2ΔN/PC7↓) would be predicted. To test this hypothesis, the different T2 transfectants were treated with BFA to inhibit the arrival of newly synthesized proteins at the cell surface. The decay of the pre-existing pool of surface MHC I was then analyzed by flow cytometry. As can be seen in Fig. 5A, the survival of surface HLA-B51 is almost identical among T2(TAPwt), T2(1-2ΔN) and T2(1-2ΔN/FUR↓). BFA treatment for 16 h reduced the mean fluorescence intensity (MFI) of surface-stained MHC I in these three cell lines by ~40%. In contrast, in T2(1-2ΔN/PC7↓) cells the MFI was reduced by >65%, showing that HLA-B51 indeed turned over more rapidly in this cell line.

Furthermore, we analyzed whether the recovery of HLA-B51 surface levels after a short acid treatment (to remove pre-existing extracellularly exposed MHC I) would be affected in T2(1-2ΔN/PC7↓). In these experiments, we observed similar kinetics for the reappearance of surface MHC I complexes in T2(TAPwt), T2(1-2ΔN/PC7↓), T2(1-2ΔN/FUR↓), and T2(1-2ΔN/PC7↓) cells, as shown in Fig. 5B.

**FIGURE 5.** Survival, recovery, and recycling of MHC I-peptide complexes on the surface of T2(1-2ΔN) cells silenced for furin or PC7. A, Survival of surface-MHC I. Transfectants were treated with BFA for 0, 2, 4, 8, and 16 h before MHC I surface levels were assessed by flow cytometry using the Ab 4E. Grading of the obtained histograms from light gray (0 h) to black (16 h) indicates the different incubation times (left panel). Results are presented as a percentage of reduction in MFI at different hours compared with the MFI at 0 h (right panel). B, Surface recovery of MHC I. Cells were acid washed to remove assembled surface MHC I and then incubated at 37˚C for 0, 1, 2, and 4 h. Levels of surface HLA-B51 were determined by flow cytometry using the mAb 4E. Grading of the obtained histograms from light gray (0 h) to dark gray (4 h) indicates the different incubation times. Black histograms represent MHC I surface staining of untreated control cells (left panel). Results are depicted as percentage of increase in MFI at different hours compared with the MFI of untreated transfectants (right panel). C, Localization of MHC I in endolysosomal compartments. T2(TAPwt), T2(1-2ΔN), T2(1-2ΔN/FUR↓), and T2(1-2ΔN/PC7↓) were fixed, permeabilized, and stained with Abs specific for HLA-B51, EEA1 (marker of early endosomes) or Lamp1 (marker of late endosomes/lysosomes). Depicted are overlays of immunofluorescence stainings of HLA-B51 (green)/EEA1 (red) (upper row) and HLA-B51 (green)/Lamp1 (red) (lower row). Nuclei are visualized by DAPI (blue). Scale bars indicate 10 μm. To quantify the degree of relative colocalization, we obtained image data from 20–30 cells as described in Materials and Methods. D, Endocytic recycling of MHC I. Expression of surface HLA-B51 in the presence or in the absence of primaquine was analyzed by flow cytometry. Results are plotted as percentage of reduction of the MFI compared with the MFI of the control experiments. The depicted histogram summarizes the results of three independent experiments.
compartments are up to 3-fold higher in T2(1-2ΔN/FUR1). Strikingly, in the case of T2(1-2ΔN/PC7)) cells, the recovery remained substantially below that of the other T2 transfectants, demonstrating that PC7 is essential for a normal accumulation rate of MHC I at the plasma membrane in these cells (Fig. 5B). In line with our data from Fig. 4, we interpret this delay to reflect the loss of suboptimally loaded MHC I by lysosomal degradation. To explore whether post-ER MHC I molecules are enriched in endolysosomal compartments of T2(1-2ΔN/PC7) cells, we analyzed colocalization of HLA-B51 with early endosomes and late endosomes/lysosomes by immunofluorescence microscopy (Fig. 5C). T2(TAPwt), T2(1-2ΔN), T2(1-2ΔN/FUR1), and T2(1-2ΔN/PC7) were permeabilized and stained with Abs specific for HLA-B51, EEA1 (marker of early endosomes) or Lamp1 (marker of late endosomes/lysosomes). In the case of T2(TAPwt), T2(1-2ΔN), and T2(1-2ΔN/FUR1), HLA-B51 showed an overlap with early endosomes and late endosomes/lysosomes in the range of 3% (EEA1) and ≤1% (Lamp1), respectively. In contrast, the values of colocalization of HLA-B51 with EEA1 and Lamp1 containing compartments are up to 3-fold higher in T2(1-2ΔN/PC7), suggesting an increased endolysosomal localization of post-ER MHC I in the absence of PC7 (Fig. 5C). To assess whether internalization from the cell surface and retention or degradation in endosomes or lysosomes leads to the enhanced loss of surface-expressed HLA-B51 in T2(1-2ΔN/PC7) cells, we analyzed MHC I surface expression in the presence or in the absence of primaquine (Fig. 5D), which inhibits recycling of endocytosed proteins including HLA-B51 back to the cell surface. As seen in Fig. 5D, T2(TAPwt), T2(1-2ΔN), and T2(1-2ΔN/FUR1) cells show up to 28% reduction of surface-exposed HLA-B51 in the presence of primaquine, whereas the drug-mediated reduction of surface MHC I is much less pronounced (12%) for T2(1-2ΔN/PC7) cells. We speculate this to reflect the higher degree of disintegration of MHC I (i.e., the irreversible dissociation of MHC H chain and β2-microglobulin) in T2(1-2ΔN/PC7) cells, because in this study the PC7-dependent rescue mechanism does not act to preserve suboptimally loaded HLA-B51. Our findings in Fig. 5C and 5D suggest that in T2(1-2ΔN/PC7) cells an increased amount of post-ER HLA-B51 is enriched in endolysosomal compartments, and these molecules enter the endosomal recycling pathway to the plasma membrane, to a smaller extent.

Peptide exchange along the exocytic pathway is likely to underlie the observed stabilization of MHC I in T2(1-2ΔN) cells. Thus, the presented peptide repertoire would be predicted to differ significantly between T2(TAPwt) and T2(1-2ΔN) on the one hand and between T2(1-2ΔN) and T2(1-2ΔN/PC7) on the other hand. To address this issue directly, peptides were acid eluted from immunopurified surface-HLA-B51-complexes (Fig. 6A) and their spectra were analyzed by MALDI-MS (Fig. 6B). Comparison of the set of isolated peptides was conducted on the basis of their molecular mass in the range of 800–1350 Da (Fig. 6B, I–IV) and controlled by a mock elution experiment (Fig. 6B, V). The spectra show that the overall distribution of masses is comparable for T2(TAPwt), T2(1-2ΔN), T2(1-2ΔN/FUR1), and T2(1-2ΔN/PC7) (Fig. 6B, I–IV). However, in the spectra derived from T2(1-2ΔN) and T2(1-2ΔN/FUR1) cells (Fig. 6B, II and III), which are very much related, a collection of additional peptide peaks (signals: a–l) in the range of 1032–1298 Da are present (Fig. 6B, II and III, indicated by arrows), which are clearly missing or strongly reduced in the profile of T2(TAPwt) cells (Fig. 6B, I). In turn, only few of the

**FIGURE 6.** Profile of MHC I peptide complexes on the surface of T2(1-2ΔN) cells silenced for furin or PC7. MALDI-MS profiles of self peptides eluted from surface HLA-B51 immunopurified with 4E Ab from 2 × 10^6 T2(TAPwt) (I), T2(1-2ΔN) (II), T2(1-2ΔN/FUR1) (III) and T2(1-2ΔN/PC7) (IV) cells, respectively. As a control, 4E-conjugated sepharose beads were acid eluted (V). A. Successful isolation of surface HLA-B51 was controlled by Western blot analysis (left panel), and the obtained MHC I signals were quantified by densitometric scanning (right panel). Four different exposures were taken to ensure linearity. The obtained MHC I signals from T2 (TAPwt) were set to 100%. B. The mass spectra were visualized and analyzed using flexAnalysis software. Data were normalized to the relative amount of immunopurified HLA-B51 molecules. Spectra are representative of two independent peptide preparations. Differential peptide peaks (signals: a–z) are indicated by arrows (newly appearing signals), asterisks (reduced/disappearing signals), and boxes (increased signals).
peaks (signals: u–w) of the T2(TAPwt) spectrum were undetectable within the profiles of T2(1-2ΔN) and T2(1-2ΔN/FUR↓). We note that this partial overlap of the peptide spectra is expected, as the loss of quality control function in the PLC will not abolish the occasional binding of abundant high-affinity peptides in the ER. In fact, only the bias in loading toward such ligands would be predicted to be impaired. However, the additional peaks in the spectra of T2(1-2ΔN) and T2(1-2ΔN/FUR↓) strongly suggest that in this study a certain population of HLA-B51 molecules is stably loaded with peptide ligands that apparently do not traverse, or are ignored by, the MHC I pathway in T2(TAPwt). Hence, they are likely to represent the mass-spectrometric signature of peptide-exchange-mediated post-ER stabilization of MHC I.

Upon comparing the peptide peaks of the different T2(1-2ΔN) transfectants, we noted that about half of the peptide population that was specifically detected for T2(1-2ΔN), and T2(1-2ΔN/FUR↓) disappears from the T2(1-2ΔN/PC7↓) spectrum (signals: a–c and f–j; Fig. 6B, IV), and that some of the prominent peaks of T2(1-2ΔN) and T2(1-2ΔN/FUR↓) show reduced (Fig. 6B, indicated by asterisks) or increased signal strength (signals: x–z; Fig. 6B, indicated by boxes) in the profile of T2(1-2ΔN/PC7↓). We also noted that, in the range of 954–1174 Da, a new collection of prominent peptide peaks were visible (signals: m–t) that appear in neither T2(TAPwt) nor T2(1-2ΔN) or T2(1-2ΔN/FUR↓), suggesting that in T2(1-2ΔN/PC7↓) cells the entry of HLA-B51 into the lysosomal pathway (Fig. 4) might also contribute to the formation of stable MHC I peptide complexes that escape to certain extent lysosomal degradation (Figs. 4, 5) and are transported back to the cell surface.

Our findings provide evidence that the absence of PLC-mediated quality control of MHC I loading and/or lack of PC7 causes the presentation of an altered MHC I peptide repertoire on the cell surface.

**PC7 mediates the liberation of epitopes from exogenous peptide precursors**

We decided to analyze the contribution of PC7 to MHC I Ag processing by a cell biologic assay, in which we used a defined 15-mer peptide of EBV glycoprotein B (EBVgB, GP110), which is a well-known target substrate of pPCs (26, 27). The EBVgB-peptide gB427-441 (LRRRRR/DAGYNTPP, sequence in single-letter code) contains the pPC-cleavage motif—(R-X-K/R-R-) confirmed by ProP 1.0 analysis (28)—conserved among most herpes virus gBs (29) (positions 3–6, indicated in bold letters and by a down arrow at the cleavage site) as well as a nonameric peptide (downstream the cleavage site, positions 7–15, indicated by italics) with sequence properties of a certain population of HLA-B51 binding ligand (SYFPEITHI 1.0-score: 24) (30). For our studies, alanine at position 11 of gB427-441 was replaced by tyrosine to enable radioiodination of the pPC-substrate (RRRR-gB, LRRRRR/DAGYNTPP, replacement indicated by underlined letter). After SYFPEITHI 1.0 (30) and ProP 1.0 (28) analysis of RRRR-gB, we obtained results as good as for the original peptide. For the control experiments, we used a related gB-peptide (RRAR-gB) in which the basic pPC-cleavage motif was destroyed by replacement of arginine at position 5 with alanine (LRRRAR/DAGNYTPP, replacements indicated by underlined letter).

The experimental setup of our analysis is based on two observations. First, exogenous applied peptide derivatives often used as substrate-based inhibitors reach pPC-containing cell compartments (31). Second, T2 cells and T2 transfectants are able to process and present exogenous peptide substrates from fluid phase by MHC I (32, 33) through a cellular pathway that is apparently independent of TAP, but requires vesicular transport between secretory compartments (34), indicating that such Ag substrates could serve in T2 cell lines as a source for an alternate MHC I presentation pathway independent of cytosol and peptide transporter (35–37). Most interestingly, studies on TAP-negative APCs suggested that processing of exogenous peptides for presentation by MHC I involves post-Golgi peptide exchange (38). However, whether and to what extent pPCs are involved in this pathway is not known. To analyze the involvement of PC7 in the generation of MHC I peptide complexes, radiolabeled peptide substrates with or without pPC cleavage site (RRRR-gB and RRAR-gB) were incubated with the different T2 transfectants for 4 h at 30°C in FCS-free medium. For the isolation of MHC I molecules, cells were extensively washed and lysed in n-octylglucoside. Lysates were incubated in the cold with 4E Ab coupled to CNBr-activated sepharose. Immuno-isolated complexes were eluted, and the amount of radioactive peptides bound to immuno-isolated MHC I was determined by γ-counting. As seen in Fig. 7, significant amounts of radiolabeled peptide bound to MHC I could be detected when the T2 transfectants were incubated with the pPC-cleavable peptide RRRR-gB and not with the uncleavable peptide RRAR-gB. This finding implies that pPC cleavage is essentially required for the EBV-B51 presentation of the gB-peptide. In the case of T2(1-2ΔN) and T2(1-2ΔN/FUR↓), the amount of radiolabeled MHC I-bound peptides was ~3–4-fold higher when compared with the situation of the T2 transfectant expressing TAPwt. This is expected because the stable MHC generated in T2(TAPwt) cells is less likely to exchange its high affinity ligands. Strikingly, the amount of radiolabeled peptides bound by MHC I in T2(1-2ΔN/PC7↓) reached only ≤25% of that found for T2(1-2ΔN) and T2(1-2ΔN/FUR↓), demonstrating that PC7 is essential for efficient loading of an epitope that requires prior pPC-mediated liberation from a precursor.
Our findings demonstrate that in a situation in which the PLC quality control function fails, PC7 and not furin is involved in the increased processing of exogenous MHC I-precursor peptides that contain a pPC cleavage site in combination with a suitable downstream MHC I epitope.

Discussion

Stability of the interaction of peptides with MHC I is highly relevant for their suitability as an effective T cell epitope and in turn a successful immune response. To achieve this stability is the major function of the PLC (1), and the amount of suboptimally loaded and structurally fragile MHC I drastically increases when this quality control checkpoint does not work properly. Defective PLCs, which lack their quality control function are observed in cells expressing mutants of tapasin or TAP or do not express these central constituents of the complex at all (2–4). Because tumors of diverse tissue origin tend to radically downmodulate or even completely lose expression of tapasin or TAP, poor quality of MHC I loading is a physiologic scenario with potentially important immunologic consequences for the pathology of cancer. Furthermore, recent studies have revealed different examples of viral interference with the recruitment of MHC I into the PLC (7, 39), degradation or inhibition of PLC components, or suppression of the quality control function of the complex (5, 6). The formation of unstable MHC I molecules was observed in many of these studies (5–7), and even tapasin-independent alleles displayed enhanced thermolability in the context of expression of a viral TAP inhibitor (4). Thus, the way in which cells deal with a malfunctioning PLC (i.e., whether they succeed in rescuing unstable MHC I and ensure the surface presentation of relevant Ags) is likely to be an important factor in the outcome of pathologic processes in vivo.

Given this, a second backup mechanism along the secretory route aiming to recover MHC I molecules that carry low-affinity peptide cargo prone to dissociate, and thus of predictably low immunogenicity, would be intuitively plausible. Our finding that the rescue of unstable HLA-B51 occurs in a post-ER compartment [also by Leonhardt et al. (3)] strongly suggests the underlying mechanism to involve peptide-exchange. The reason is that acid-catalyzed dissociation of peptides and their reassociation with MHC I has been observed at pH conditions corresponding to the increasingly acidic environment along the endocytic and exocytic pathways (40–42).

Peptide exchange, in turn, would be predicted to occur preferentially at predestabilized MHC I peptide complexes, thus explaining why the pPC-dependent mechanism is so much more effective in T2(1–2ΔN) than in T2(TAPwt) cells. Furthermore, the existence of such a mechanism, as well as its breakdown in T2(1–2ΔN/PC7) cells, is also directly supported by the qualitative and quantitative changes in the MALDI-mass spectra characteristic for the four analyzed T2 transfectants (Fig. 6B). Finally, our finding (based on pharmacologic and genetic evidence) that this pathway essentially requires PC7 activity, argues that in T2(1–2ΔN) cells an already fragile MHC I population acquires pPC-dependent ligands upon further acid destabilization in the late secretory compartments in which PC7 resides (23). Interestingly, the capacity of MHC I to bind peptides at acidic pH corresponding to the TGN has been shown to vary among different alleles, some of which even display superior binding at acidic versus neutral pH (29). Routing of HLA-B51 to lysosomes in T2(1–2ΔN/PC7) cells, however, obviously fails to efficiently rescue the molecule, which can be likely attributed to the substantially lower pH in these compartments preferring irreversible dissociation of MHC I rather than promoting peptide exchange. In addition, we note that our model is in line with several reports that demonstrate that pPC-dependent peptides can be presented by MHC I (3, 8, 9, 43–45) (some of the mentioned studies describe loading of pPC-dependent epitopes in the TGN during secretion, whereas loading is thought to occur via endosomal recycling in others). Unfortunately, most of these reports did not fully clarify (or did not address) which particular pPCs was acting in their experimental system, and all above studies focused at the generation of particular epitopes, but not on bulk-loading of naturally occurring intracellular peptides. In this context, it should be mentioned that none of the commercially available pharmacologic pPC inhibitors displays sufficient specificity to convincingly discriminate between furin and PC7 activity. Also, vaccinia virus-driven overexpression of pPCs used in some of these studies (46) has been shown to be prone to cause cleavage artifacts, which is an issue that may be particularly critical when T cell activation is used as a readout because few cleavage events might be fully sufficient to generate enough MHC-peptide complexes to drive profound CTL-mediated target cell lysis. Consequently, silencing by RNA interference is probably the most specific approach to assess functional differences between individual pPCs when working with human cells.

We also note that the peptide-exchange mechanism we propose provides an elegant explanation of why the peptides generated by PC7 can stabilize HLA-B51 in T2(1–2ΔN) cells, whereas the ligands available in the ER obviously cannot. Certainly, it is highly unlikely that the pPC-generated peptide pool has a generally higher affinity for MHC I as the peptides generated by the proteasome. Rather, it is plausible to assume that it is the acidic environment in the TGN that confers the high stringency on MHC I loading and allows only optimal peptide ligands to bind stably (i.e., not to dissociate).

However, such a pPC-dependent second checkpoint would have to introduce relevant epitopes into the MHC I-presented repertoire to become immunologically significant. In this context, it is important to note that many viral proteins are processed by pPCs (e.g., diverse fusion proteins including those from influenza A virus, respiratory syncytial virus, Ebola virus, severe acute respiratory syndrome virus, human CMV, hepatitis B virus, HIV) (13, 26, 47, 48). It is obvious that under conditions of an acute infection these viral proteins will become highly abundant in the secretory compartments, and it has been shown that MHC I-presented viral Ags can originate from pPC-processed viral polypeptides (49). Thus, the pPC-dependent rescue of MHC I complexes could force the surface presentation of important viral epitopes with significant immunologic consequences. Because severe viral interference with the assembly and function of the PLC has been observed (5–7, 50), this pPC-dependent pathway might allow for a functional rescue of MHC I-mediated Ag presentation and consequently lead to an enhanced clearance of infected cells. Thus, we propose that this alternative PC7-dependent pathway of MHC I loading might be part of a cellular counter strategy that undermines viral efforts to block the Ag presentation machinery.

Similar considerations may also apply for anticancer immune responses, because some tumor-associated Ags, such as gp100, are also cleaved by pPCs (51). This should be considered when pPC inhibitors are used in the context of cancer therapies as suggested by several laboratories, based on findings that pPCs promote tumor growth and metastasis (52). However, we note that PC7 could also protect tumors from NK cell-mediated attack by securing high MHC I levels despite low TAP or tapasin function in malignant cells.

One striking point is the magnitude of PC7-mediated MHC I rescue that we observed. To our knowledge, the contribution of no single protease in the MHC I pathway other than the proteasome (53) and ERAAP (54) has been reported to measurably enhance overall MHC I surface levels, although many accessory proteases such as TPP2 (55), leucine aminopeptidase (56), puromycin-sensitive aminopeptidase (57), thimet oligopeptidase (58), and bleomycin...
striking differences in stability (Figs. 3 presented in cells harboring malfunctioning PLCs as a consequence loading will make a substantial contribution to the repertoire of Ags enormous potency strongly suggests that PC7-mediated MHC I hydrolase (59) have been analyzed in knockout systems. This in this sense. Of note, HDA has a broad inhibitory capacity requirement for PC7, but not furin, more than half of the peptide neutral in this sense. Of note, HDA has a broad inhibitory capacity against most pPCs (including PC7), blocks furin activity efficiently in vivo, and actually has a >10-fold higher potency toward furin than toward PC7 (24). Further corroborating the selective re-

The MHC I-loading experiments in Fig. 7, in which we used exogenous oligopeptides of a natural pPC target (EBVgB) containing a suitable MHC I epitope downstream the proprotein cleavage site, bypassed the need for further C-terminal substrate (e.g., carboxypeptidase D) and thus clearly created an ideal model substrate situation for PC7 and MHC I. Nevertheless, our studies revealed that the observed increased MHC I loading of processed ligands in T2 transfectants with defective PLC is essentially dependent on PC7 and not on furin. In accordance with our other findings, this strongly supports the idea that PC7 is able to produce stable MHC I ligands directly from appropriate precursor substrates and could function as one of the main executors within this pathway. Although several studies described MHC I loading of exogenous peptide substrates in T2 cells (32–34), it is not clear where exogenous ligands are processed and gain access to MHC I. One of the possible scenarios that have been discussed allows processing of exogenous substrates and peptide binding to MHC I in post-Golgi compartments (64). Therefore, it is tempting to speculate that for exogenous oligopeptides that fit the substrate requirements of pPCs, PC7 could play an important role in this supplemental pathway, which seems to work much more efficiently under conditions in which the PLCs are unable to generate stable MHC I peptide complexes.

Interestingly, PC7 is known to display a widespread distribution in different organs and cell types (12), including expression in professional APCs such as dendritic cells and macrophages (65; data not shown). The PC7-dependent rescue pathway may thus be a ubiquitous backup module present in all cells, ready to handle otherwise detrimental attacks of viral effectors against PLC-mediated MHC I loading.

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Disclosures
The authors have no financial conflicts of interest.

References

hydrolyse (59) have been analyzed in knockout systems. This enormous potency strongly suggests that PC7-mediated MHC I loading will make a substantial contribution to the repertoire of Ags presented in cells harboring malfunctioning PLCs as a consequence of pathologic processes also in vivo.

One of the most intriguing findings of this study is that only PC7 is required for post-ER rescue of unstable MHC I, whereas furin appears to be completely dispensable. This is supported by the striking differences in stability (Figs. 3C, 3D, 5A) and steady-state levels (Fig. 2C) of surface-MHC I observed between T2(2A/N-FUR) and T2(2A/N-PC7). In fact, that these levels completely lose sensitivity toward the pPC inhibitor HDA in cells lacking PC7 (Fig. 2D) underscores the exclusive involvement of this particular pPC, whereas a practically complete furin knockdown is totally neutral in this sense. Of note, HDA has a broad inhibitory capacity against most pPCs (including PC7), blocks furin activity efficiently in vivo, and actually has a >10-fold higher potency toward furin than toward PC7 (24). Further corroborating the selective re-

Alternatively, it could be a spatial separation of furin and PC7 that underneath the functional differences of these two pPCs in Ag present-
ation. In accordance with the results of our immunofluorescence experiments depicted in Fig. 1D, studies by Wouters et al. (23) suggest that the mature active forms of furin and PC7 display distinct subcellular distributions, with furin predominantly residing in the TGN and PC7 being localized to TGN-derived secretory vesicles. Most interestingly, secretory vesicles budding from the trans-cisternae of the Golgi have been shown to be more acidic than the trans-

Golgi in islet cells, and these differences in pH correlate with the processing of a pPC substrate (61). Hence, it is possible that only the PC7-containing late secretory compartments provide the pH necessary to catalyze efficient peptide exchange in HLA-B51, whereas furin-containing compartments do not. Alternatively, PC7-containing secretory vesicles may be the superior environment for generation and/or survival of MHC I ligands, because of the presence or absence of downstream proteases further processing theodomains liberated by pPCs. This is an important issue, because MHC I-bound peptides are much shorter in length than most of theseodomains;


