The structure and location of SIMP/STT3B account for its prominent imprint on the MHC I immunopeptidome

Étienne Caron*, Renée Charbonneau*, Gabrielle Huppé, Sylvie Brochu and Claude Perreault

Institute of Research in Immunology and Cancer, University of Montreal, Casier Postal 6128, Succ. Centre-ville, Montreal, Quebec H3C 3J7, Canada

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Abstract

Proteins show drastic discrepancies in their contribution to the collection of self-peptides that shape the repertoire of CD8 T cells (MHC I self-immunopeptidome). To decipher why selected proteins are the foremost sources of MHC I-associated self-peptides, we chose to study SIMP/STT3B because this protein generates very high amounts of MHC I-associated peptides in mice and humans. We show that the endoplasmic reticulum (ER)-associated degradation pathway and MHC I processing intersect at SIMP/STT3B. Relevant key features of SIMP/STT3B are its lysine-rich region, its propensity to misfold and its location in the ER membrane in close proximity to the immunoproteasome. Moreover, we show that coupling to SIMP/STT3B can be used to foster MHC I presentation of a selected peptide, here the ovalbumin peptide SIINFEKL. These data yield novel insights into relations between the cell proteome and the MHC I immunopeptidome. They suggest that the contribution of a given protein to the MHC I immunopeptidome results from the interplay of at least three factors: the presence of degrons (degradation signals), the tendency of the protein to misfold and its subcellular localization. Furthermore, they indicate that substrates of the ER-associated degradation pathway may have a prominent imprint on the MHC I self-immunopeptidome.

Introduction

The role of classical MHC class I molecules is to display a small sample of the cell proteome, referred to as the immunopeptidome, for scrutiny by CD8 T cells (1–3). Under basal conditions, the immunopeptidome is composed of short peptides, typically nonamers, derived from ‘self’-proteins. The self-immunopeptidome molds T cell development and homeostasis by regulating intrathymic positive and negative selection, as well as peripheral T cell survival and expansion (4). Moreover, those among self-peptides that display inter-individual polymorphism (i.e. minor histocompatibility antigens) represent a major barrier in transplantation (5, 6), while those that are expressed selectively or preferentially on neoplastic cells are potential targets for cancer immunotherapy (7). Studies based on sequencing of MHC-associated peptides by mass spectrometry suggest that some proteins supply much more MHC-associated peptides than others (8, 9). To decipher why selected proteins are foremost sources of MHC I-associated self-peptides, we chose to study SIMP/STT3B because this protein displays an ‘extreme phenotype’ in that it generates exceedingly high amounts of MHC I-associated peptides in mice and humans. In H2k mice, ~1% of D8 molecules on spleen cells are occupied by KAPDNRETL, a peptide derived from SIMP/STT3B (10–13; Fig. 1). Likewise, following comprehensive proteomic analyses of the HLA-B*1801 immunopeptidome, the SIMP/STT3B DERVFVALY peptide emerged as one of the most abundant peptides (9). Moreover, we recently found that the SIMP/STT3B NLYDKAGKV peptide was a major constituent of the HLA-A2 immunopeptidome (C. Perreault, unpublished observation). SIMP (source of immunodominant MHC-associated peptides) was originally identified as the gene encoding the immunodominant B6dom1/H7a minor histocompatibility antigen (13). SIMP was later shown to represent one of the two functional homologs of the yeast STT3 protein found in multi-cellular eukaryotes and was thus named STT3B (15). STT3 homologs, large polytopic glycoproteins located

*These authors contributed equally to this work.

Correspondence to: C. Perreault; E-mail: c.perreault@videotron.ca

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in the endoplasmic reticulum (ER) membrane, contain the active site of the oligosaccharyltransferase complex that catalyzes N-linked glycosylation of nascent proteins in the lumen of the ER (16).

Current evidence suggests that the MHC I immunopeptidome derives from the rapid degradation of newly synthesized proteins (17, 18). Rapidly degraded proteins are of two types: normal short-lived proteins and defective ribosomal products (DRiPs) (19–21). As defined by Yewdell (22), DRiPs 'include properly translated but misfolded proteins and misbegotten polypeptides resulting from mistakes in the fidelity of transcription or translation'. The processing of antigens to generate MHC I-associated antigenic peptides occurs predominantly, though not exclusively, by proteasome-mediated degradation (3, 23). Proteasomes are present in the cytoplasm and nuclei of all eukaryotic cells, but with highly variable relative abundance within those compartments (24). Several studies have shown proteasome enrichment in the vicinity of the microtubule organizing center (MTOC) and on the cytosolic side of the ER (25–27). This raises the interesting possibility that subcellular localization of proteins might influence their proteasomal degradation. Because of the lack of proteasome inside the ER (28), degradation of ER proteins must be preceded by their retrotranslocation (or dislocation) from the ER to the cytosol for degradation by the proteasome in a process called endoplasmic reticulum-associated degradation (ERAD) (29). In our quest to understand why STT3B has a prominent contribution to the MHC I immunopeptidome, we found that MHC I processing and ERAD intersect at STT3B. The results of our studies suggest that three features render STT3B a major ERAD substrate: its location in the ER membrane, its propensity to form DRiPs and its conserved lysine-rich region that functions as a degradation signal (degron). Furthermore, we found that coupling to STT3B can be used to increase MHC I presentation of a selected peptide.

**Methods**

**Plasmid constructs**

The STT3B construct was generated by inserting the coding sequence of mouse STT3B (13) in pcDNA3 vector (Invitrogen, Burlington, Ontario, Canada). EGFP-STT3B and c-myc-STT3B were constructed by inserting the STT3B open reading frame (ORF) into pEGFP-C2 (Clontech, Mountain View, CA, USA) and pCMV-Tag3C (Stratagene, Cedar Creek, TX, USA), respectively. STT3B-FLAG was produced following PCR amplification to create a mutation into the stop codon, and then inserted into the pFLAG-CMV5a vector (Sigma-Aldrich, Oakville, Ontario, Canada). The EGFP-STT3B construct
encoding SIINFEKL instead of KAPDNRETL peptide (EGFP-STT3B-SIINFEKL) was inserted into pEGFP-C1 (Clontech). EGFP-STT3B-SIINFEKL was created by PCR using a reverse primer extended by the SIINFEKL coding sequence to produce STT3B-780-SIINFEKL. STT3B790-823 was amplified and inserted with STT3B-780-SIINFEKL in the same ORF into pEGFP-C1. EGFP-SIINFEKL was created following insertion of the SIINFEKL coding sequence into pEGFP-C1. The STT3B790-823 coding sequence was excised to produce EGFP-STT3B790-823-SIINFEKL. To create EGFP-STT3B790-823-SIINFEKL, the STT3B611 and STT3B663-823 coding regions were amplified by PCR and inserted in the same ORF into the pEGFP-C1 vector. EGFP-OVA-STT3B790-823 was created following insertion of ovalbumin peptide (OVA)61-386 and STT3B790-823 in the same ORF into pEGFP-C1 vector. Hemagglutinin (HA)-ubiquitin was a gift from S. Meloche (Institute of Research in Immunology and Cancer, University of Montreal, Quebec, Canada). The pIRE2-EGFP cloning vector (Clontech) was used to insert the OVA and SIINFEKL ORF excised from pcDNA3.1 (kindly provided by K. Rock, Department of Pathology, University of Massachusetts Medical School, Worcester, MA, USA) and STT3B-SIINFEKL from pEGFP-C1. To create OVA-STT3B790-823 into the pIRE2-EGFP vector, the OVA coding sequence was amplified by PCR to create a mutation into the stop codon, and then inserted with the STT3B790-823 coding region in the same ORF. To generate EGFP-SIINFEKL, two oligonucleotides encoding the SIINFEKL peptide were annealed and inserted into pEGFP-C1. The STT3B790-823 Coding sequence was added to EGFP-SIINFEKL to generate EGFP-SIINFEKL-STT3B790-823.

**Reagents and antibodies**

Clipso-lactacystin β-lactone was purchased from Calbiochem (San Diego, CA, USA), 3-methyl-adenine and bafilomycin A1 from Sigma-Aldrich and recombinant human IFN-γ from Peprotech (Rocky Hill, NJ, USA). Rabbit polyclonal antibody against the following molecules was used: anti-calnexin and anti-calreticulin from Stressgen (Victoria, British Columbia, Canada), anti-c-myc from Abcam (Cambridge, MA, USA), anti-calnexin antibodies. The reaction was developed by using the ECL + system (Amersham Pharmacia Biotech).

**Cell culture and transfection**

HeLa-Kb and COS-7-Kb cells were kindly provided by T. van Hall (Leiden University Medical Center, Leiden, The Netherlands) and K. Rock, respectively. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Transient transfection was performed according to the manufacturer’s instructions using FuGENE 6 transfection reagent (Roche) for COS-7, COS-7-Kb and HEK 293 cells, and Lipofectamine reagent (Invitrogen) for HeLa-Kb cells.

**Cytofluorography**

Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software and sorted on a FACSVantage SE system with FACS DIVA option (BD Biosciences, Mississauga, Ontario, Canada). Cell-surface Kb-SIINFEKL labeling was carried out as described using Alexa-labeled 25-D1.16 mAb (30).

**Immunocytochemistry**

Cells on coverslips were fixed in 4% formaldehyde in PBS for 30 min or in methanol at −20°C for 20 min, then washed twice in PBS and permeabilized with 0.1% triton X-100 in PBS for 30 min at room temperature (RT). Cells were then washed thrice in PBS, blocked with 10% FBS in PBS for 1 h and incubated with primary antibody diluted in 1% BSA 0.01% triton X-100 in PBS for 1 h at RT. After washing thrice in PBS, coverslips were incubated with secondary antibody diluted in 1% BSA 0.01% triton X-100 in PBS for 1 h at RT. Cells were washed five times in PBS, mounted with Vectashield with 4’,6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA, USA) and analyzed under a Zeiss LSM 510 confocal microscope (Zeiss, North York, Ontario, Canada).

**Electron microscopy**

Trypsinized cells were collected in a pellet by centrifugation at 2000 × g, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate
buffer for 1 h at 4°C, postfixed in 1% osmium acid and embedded in epon. Lead citrate-stained thin sections were examined under a Hitachi (Rexdale, Ontario, Canada) H-7500 transmission electron microscope and photographs were taken with a Hamamatsu (Markham, Ontario, Canada) digital camera model C4742-95.

Results

**STT3B enhances MHC I presentation of OVA-derived peptide**

In theory, the prominent contribution of STT3B to the immunopeptidome could be due solely to the high affinity of selected STT3B peptides for several MHC I allelic products. The STT3B-derived peptide presented by H2Db (KAPDNRETL) has indeed a high affinity for H2Db (11). However, the fact that the STT3B-derived peptide (NLKDKAGKV) presented by HLA-A2 has a low affinity for HLA-A2 (data not shown) argues against this contention and suggests that other factor(s) account for the major contribution of STT3B to the immunopeptidome. A likely explanation would be that STT3B is processed more efficiently than other proteins along the MHC I pathway. To test this hypothesis, we transfected HeLa-Kb cells with two chimeras: EGFP-SIINFEKL and EGFP-STT3B-SIINFEKL (Fig. 2A). We postulated that with these constructs, EGFP fluorescence would be proportional to the quantity of intracellular protein containing the SIINFEKL peptide. We then assessed 25-D1.16 mean fluorescence intensity (MFI) in discrete cell populations expressing various levels of EGFP intensity. This strategy allowed us to correlate the amount of Kb-SIINFEKL complexes at the cell surface with intracellular SIINFEKL. In cells expressing EGFP-STT3B-SIINFEKL, the intensity of 25-D1.16 staining increased in a linear fashion as a function of EGFP MFI up to an EGFP MFI of 80 where a plateau was reached (Fig. 2B). The notable point was that until this plateau was reached, EGFP-STT3B-SIINFEKL generated six times more Kb-SIINFEKL complexes than EGFP-SIINFEKL (Fig. 2B). Thus, the amount of Kb-SIINFEKL complexes was dramatically increased by coupling SIINFEKL to STT3B.

**STT3B tends to adopt a non-native conformation and induces ER biogenesis**

Because of growing evidence that, at least for viral proteins, DRiPs represent an important source of MHC I-associated peptides (31), we asked whether synthesis of STT3B would generate high amounts of DRiPs. DRiPs tend to adopt a non-native (misfolded) conformation and their insoluble nature makes quantitative analysis difficult and prevents precise estimation of the DRiP rate. To estimate the propensity of STT3B to generate DRiPs, we therefore adopted a two-pronged strategy based on biochemistry and cell biology.

**Biochemistry.** A general feature of misfolded proteins is that they are insoluble and resistant to extraction with relatively mild detergents (32, 33). We therefore wished to determine whether this was the case for STT3B. We extracted membrane proteins from COS-7-Kb cells transfected with c-myc-STT3B using three detergents: digitonin, CHAPS + deoxycholate and n-dodecyl-B-D-maltoside. The soluble and insoluble fractions were resolved in 8% SDS-PAGE and probed with anti-myc and anti-calnexin antibodies. Like STT3B, calnexin is an integral ER membrane protein. While calnexin partitioned in similar amounts in the soluble and insoluble fractions, STT3B mainly partitioned into the detergent-insoluble fractions (Fig. 3). Furthermore, STT3B, but not calnexin, migrated for the most part as high Mr complexes (150–250 kDa) rather than monomers (~75 kDa). These complexes are evidence of a non-native conformation (misfolded oligomers) because
STT3B is monomeric in its native membrane (34). Of note, yeast HA-tagged STT3 was also found to be poorly soluble and showed an anomalous electrophoretic migration that persisted after deglycolysation (32).

**Cell biology.** Misfolded proteins found in the ER become ERAD substrates whose disposal is limited by two bottle-necks: dislocation across the membrane and actual degradation by the proteasome (35). Inefficient proteolysis results in the formation of pericentriolar membrane-free cytoplasmic inclusions called aggresomes (36). By contrast, inefficient dislocation results in substrate accumulation in the ER, which leads to an XBP1-dependent biogenesis of ER membranes (37–40). Aggresomes and accretion of ER arrays can be detected by fluorescence and electron microscopy. To determine whether STT3B would induce formation of aggresomes or accumulation of ER membranes, we studied COS-7 cells transfected with EGFP-STT3B constructs. From 24 to 48 h after transfection, STT3B appeared in most cells in a disperse ER pattern in close proximity to calnexin and calreticulin (Fig. 4A). The STT3B pattern showed significant though not complete overlap with Sec61 (Fig. 4A) but none with COP II (data not shown). Notably, in ~40% of EGFP-STT3B-transfected COS-7 cells, fluorescence accumulated in a juxtanuclear compartment (Fig. 5A and B) not seen in cells transfected with EGFP (data not shown). Accumulation in a tight paranuclear pattern was not cell-type specific since it was also observed, albeit at lower frequency, in HEK 293 and NIH 3T3 cells transfected with EGFP-STT3B (Fig. 5B). The pattern was not EGFP dependent since it was also induced by

![Fig. 4](https://academic.oup.com/intimm/article-abstract/17/12/1583/746090) EGFP-STT3B is located in the ER. (A) COS-7 cells were transfected with EGFP-STT3B, and after 40 h were stained with antibody to calnexin, calreticulin and Sec61β, and imaged to localize EGFP-STT3B. (B) Immunoproteasome induction by IFN-γ. COS-7 cells cultured for 88 h with or without 250U/ml IFN-γ were stained for LMP2. (C) Higher magnification view of a COS-7 cell treated with IFN-γ. (D) Colocalization of EGFP-STT3B and LMP2 in IFN-γ treated COS-7 cells. Bar for A, C and D = 5 μm, and for B = 10 μm.
constructs in which STT3B was tagged with c-myc or FLAG epitopes (Fig. 5C). Juxtanuclear accumulation of EGFP-STT3B was impaired by the microtubule-depolymerizing drug nocodazole (Fig. 5D). Nocodazole-treated cells showed dispersed small protein clusters rather than the sequestration of one major juxtanuclear pattern.

Confocal microscopy revealed that three ER markers, calnexin, calreticulin and Sec61β, but not ubiquitin, concentrated to the same paranuclear compartment where STT3B accumulated (Fig. 5A). In mammalian cells, the immunoproteasome is associated primarily with the cytosolic side of the ER membrane (24, 25), and staining with anti-LMP2 antibody shows a dispersed ER-like pattern in IFN-γ-treated cells (Fig. 4B and C). Immunostaining for LMP2 disclosed a striking enrichment of immunoproteasome in the same juxtanuclear compartment where STT3B accumulated (Fig. 4D). The subcellular compartment was adjacent to the centrioles as visualized by staining of γ-tubulin (Fig. 5A). Consistent with colocalization of STT3B with ER markers, transmission electron microscopy showed bulky accumulation of anastomosing smooth ER tubules in ~30% of cells transfected with EGFP-STT3B (Fig. 6). Accumulation of ER tubules was not observed in untransfected cells (Fig. 6) or cells transfected with EGFP alone (data not shown). The latter observation indicates that

Fig. 5. EGFP-STT3B accumulates in a juxtanuclear ER compartment. (A) At 40 h after transfection with EGFP-STT3B, COS-7 cells were stained with antibody to calnexin, calreticulin, Sec61β and γ-tubulin and imaged to localize EGFP-STT3B. For ubiquitin colocalization, cells were cotransfected with HA–ubiquitin and EGFP-STT3B vectors and immunostained for HA. (B) Juxtanuclear accumulation of EGFP-STT3B in three cell lines 24 h after transfection with EGFP-STT3B. The number of cells with juxtanuclear accumulation of EGFP-STT3B is reported as a percentage of transfected cells. Six hundred cells were counted for each condition. (C) Juxtanuclear accumulation of STT3B is not EGFP dependent. At 40 h after transfection with c-myc-STT3B or STT3B-FLAG, COS-7 cells were immunostained with anti-c-myc or anti-FLAG antibody. (D) COS-7 cells were transfected with EGFP-STT3B and treated or not with nocodazole (10 μM) 6 h after transfection. Bar = 5 μm.
accumulation of STT3B leads to massive ER biogenesis and paranuclear accretion of ER membranes. On the whole, the ER compartment where STT3B accumulates shares the features of the yeast ‘ER-associated compartment’ (41) and the mammalian ‘ER quality control compartment’ (42). The prevailing view is that these ER subdomains serve as repositories for misfolded proteins that have not been translocated to the cytosol. Together, STT3B insolubility and tendency to adopt a non-native conformation (high Mr forms), and its ability to induce a massive ER biogenesis provide compelling evidence that STT3B is prone to misfolding.

STT3B is degraded by the proteasome system and not by autophagy

The major pathways for degradation of cellular constituents are autophagy and cytosolic turnover by the proteasome (43, 44). Intracellular levels of EGFP-STT3B were significantly increased following treatment with clasto-lactacystin β-lactone but not with inhibitors of autophagy (3-methyl-adenine and bafilomycin) (Fig. 7A). Thus, degradation of STT3B is mediated primarily, if not exclusively, by the proteasome.

We showed previously that when the SIINFEKL peptide is inserted into STT3B [between amino acid (aa) 769 and 779] it is efficiently processed along the MHC I pathway and presented by H2kβ (Fig. 2B). In our next experiment we wished to determine whether this processing was proteasome dependent. COS-7-Kb cells were transfected with three constructs: OVA, STT3B-SIINFEKL and an MSIINFEKL minigene. Cells were treated with citric acid to elute MHC-associated peptides, then cultured in the presence of graded concentrations of clasto-lactacystin β-lactone for 4 h and stained with 25-D1.16 antibody (Fig. 7B). Proteasome inhibition by clasto-lactacystin β-lactone induced a dose-dependent decrease of cell-surface levels of Kb-SIINFEKL complexes in cells transfected with OVA but, as expected (25), not in cells transfected with the MSIINFEKL minigene. The key finding was that in cells expressing STT3B-SIINFEKL, the generation of Kb-SIINFEKL complexes was abrogated at exceedingly low concentrations of clasto-lactacystin β-lactone. Thus, MHC I presentation of peptides embedded in STT3B is proteasome dependent.

Retrotranslocation of STT3B is coupled to its degradation and is facilitated by N-linked glycans

Proteasomal degradation of proteins present in the ER lumen requires their retrotranslocation to the cytosol (46, 47). It has
been hypothesized, however, that proteasomal degradation of integral ER membrane proteins, such as STT3B, might be initiated prior to retrotranslocation (48). Nevertheless, the predicted location of two STT3B-derived MHC I-associated peptides on the luminal side of ER membrane (Fig. 1) suggests that retrotranslocation of STT3B is important for proteasomal degradation. In many cases, dislocation of ERAD substrates is tightly coupled to proteasomal degradation and so they accumulate in the ER following proteasome inhibition (42, 49). We found that this was the case for STT3B. Indeed, following transfection with EGFP-STT3B, the percentage of cells showing juxtanuclear accumulation of fluorescence in ER arrays was more than doubled in the presence of clasto-lactacystin β-lactone (Fig. 7C). Of note, in the latter conditions, EGFP fluorescence was limited to the juxtanuclear ER arrays and was not detected in the cytosol (data not shown). Thus, as for many ERAD substrates, degradation of STT3B is linked to its retrotranslocation.

ER to cytosol dislocation of at least some glycoproteins is critically dependent on the recognition of specific glycosylation

![Image](https://academic.oup.com/intimm/article-abstract/17/12/1583/746090)
intermediates of N-linked glycans by the ER degradation-enhancing alpha-mannosidase-like protein (EDEM) lectin (50, 51). To determine whether this was the case for STT3B we expressed a mutant form of STT3B in which the region containing N-glycosylation sites (aa 612–662, see Fig. 1) was deleted. We then compared COS-7-Kb cells transfected with EGFP-STT3B-SIINFEKL versus EGFP-STT3BΔ612–662-SIINFEKL constructs. Deletion of N-glycosylation sites caused a 3-fold increase in the proportion of cells with paranuclear STT3B accretion in ER arrays (Fig. 7D) that could be due to two factors: an increased proportion of unfolded protein and decreased protein retrotranslocation. Notably, deletion of the glycosylation sequon decreased the generation of Kb-SIINFEKL epitopes by ~20% (Fig. 7E; P < 0.05, Student’s t-test). Thus, protein accumulation was due at least in part to impairment of retrotranslocation and was not due solely to an increased proportion of unfolded protein. Nonetheless, the fact that loss of N-glycosylation sites has a relatively modest impact on the generation of STT3B-derived MHC I-presented peptides suggests that N-linked glycans have a contributory but not essential role in STT3B retrotranslocation and degradation.

**STT3B retrotranslocation is a limiting step in peptide generation**

We reported in Fig. 2(B) that generation of Kβ-SIINFEKL complexes from EGFP-STT3B-SIINFEKL was a saturable process that reached a plateau when EGFP attained a MFI of ~ 80. We wished to elucidate the nature of the limiting step in STT3B processing. Cells with low and high EGFP fluorescence (Fig. 8A) were electronically sorted and the percentage of cells with EGFP accumulation in juxtanuclear ER arrays was determined by fluorescence microscopy. More than 80% of cells with high EGFP levels, but none with low EGFP levels, showed paranuclear EGFP accumulation (Fig. 8B). These data suggest that retrotranslocation is a limiting step in STT3B processing by the ERAD pathway and that ER biogenesis occurs when the ER membrane contains more STT3B than it can dislocate. The idea that STT3B is difficult to retrotranslocate but is rapidly degraded once in the cytosol is supported by two observations: (i) under no circumstances did we detect STT3B in the cytosol, even in cells with massive accumulation of EGFP-STT3B in the ER (e.g. in cells treated with clasto-lactacystin β-lactone as in Fig. 7C) and (ii) in vivo STT3B accumulates only in non-ubiquitinated form(s) (Fig. 5A). No plateau in the generation of Kβ-SIINFEKL complexes was observed with the EGFP-OVA-STT3B790–823 construct (Fig. 8C). The latter chimera contains only a 34-aa sequence from STT3B and its product diffuses in the cytosol (data not shown). The absence of a plateau with the latter construct suggests that there is no additional bottleneck involved in MHC processing of SIINFEKL downstream of retrotranslocation (e.g. proteolysis by the proteasome, interaction with TAP and MHC binding).

Fig. 8. Correlation between protein levels, juxtanuclear pattern and cell-surface levels of Kβ-SIINFEKL complexes. (A) HeLa-Kb cells transfected with EGFP-STT3B-SIINFEKL construct were assessed for EGFP fluorescence and labeling with 25-D1.16 mAb (Kβ-SIINFEKL MFI) as in Fig. 2(C). (B) Cell populations with low or high EGFP fluorescence were sorted on a FACSVantage SE system and prepared for fluorescence microscopy. The percentage of cells showing juxtanuclear accumulation of EGFP was calculated after counting 200 cells. (C) HeLa-Kb cells transfected with two EGFP constructs were assessed for EGFP fluorescence and labeling with 25-D1.16 mAb.
IFN-γ enhances generation of K<sup>b</sup>-SIINFEKL complexes
in cells transfected with EGFP-STT3B-SIINFEKL

One striking feature of STT3B is its subcellular localization in close proximity to the proteasomes. Being located in the ER membrane, STT3B locates in the vicinity of the immunoproteasome which is primarily found on the cytosolic side of the ER (24, 27; Fig. 4C and D). Furthermore, STT3B can induce ER biogenesis and accumulation of ER arrays in close proximity to the MTOC (Fig. 5A), a major site of proteasomal degradation in the cytosol (25).

To determine whether subcellular localization of STT3B influenced its processing along the MHC I pathway, we first asked whether preventing recruitment of STT3B to the MTOC would influence the generation of MHC I-associated peptides. We showed earlier that among cells expressing EGFP-STT3B-SIINFEKL, >80% of ‘EGFP high’ cells showed juxtanuclear accumulation of EGFP* ER arrays (Fig. 8B). Treatment with nocodazole completely abrogates formation of this paracentriolar compartment (Fig. 9A). The microtubule-depolymerizing effect of nocodazole treatment does not abrogate ER biogenesis but prevents recruitment of ER arrays to the MTOC (Fig. 5D). Thus, we asked whether nocodazole would influence the amount of K<sup>b</sup>-SIINFEKL complexes presented at the surface of EGFP high cells (i.e. the cells with paracentriolar accumulation of ER arrays) and found that it was not the case (Fig. 9B). Preventing recruitment of ER arrays towards the MTOC with nocodazole had no discernible effect on the generation of K<sup>b</sup>-SIINFEKL complexes.

We next wished to evaluate whether the close proximity between STT3B and the immunoproteasome might be functionally important for STT3B processing. We therefore asked whether increasing the levels of immunoproteasome in cells expressing STT3B-SIINFEKL would influence the MHC presentation of SIINFEKL. Thus, HeLa-K<sup>b</sup> cells transfected with EGFP-STT3B-SIINFEKL were treated with IFN-γ and the amount of intracellular proteins was correlated with the cell-surface density of K<sup>b</sup>-SIINFEKL complexes. As depicted in Fig. 9(C), IFN-γ treatment dramatically raised the plateau in the generation of K<sup>b</sup>-SIINFEKL complexes. Indeed, the maximal amount of K<sup>b</sup>-SIINFEKL complexes was increased by 2- to 3-fold in IFN-γ-treated cells relative to controls. The effects of IFN-γ are not limited to induction of the immunoproteasome and encompass up-regulation of molecules such as TAP and MHC I (52). However, that retrotranslocation is the sole limiting step in MHC processing of STT3B peptides and is tightly coupled to proteasome degradation (Figs 7 and 8C), strongly suggests that the effect of IFN-γ in these experiments was due to immunoproteasome-mediated enhancement of STT3B retrotranslocation.

The lysine-rich region of STT3B enhances the generation of MHC I-associated peptides

In general, ubiquitination is a prerequisite for proteasomal degradation and is initiated by formation of an isopeptide...
The prevailing view is that in cases of viral infection, virus MHC I-associated peptides derive largely if not mainly from DRiPs. Our work provides strong evidence that STT3B, a major source of MHC I-associated peptides, has a high propensity to misfold. This conclusion is based on the following evidence: the demonstration that STT3B is resistant to extraction with several detergents and tends to form high M complexes, and the tendency of over-expressed STT3B to induce ER biogenesis. These data suggest that the MHC I self-immunopeptidome like its viral counterpart is molded by proteins with a high DRiP rate. Anatomosing ER arrays are thought to represent holding sites to which misfolded proteins are specifically diverted so as not to interfere with normal cellular functions (41). These structures form when mutant membrane proteins accumulate in the ER due to defects in their ability to be dislocated to the cytosol (54, 55). However, in some situations ER biogenesis may also be induced by the increased accumulation of non-mutant resident proteins (40). While the high DRiP rate of STT3B leaves practically no doubt, further studies are needed to determine whether all non-mutant proteins that can induce ER biogenesis share a similar DRiP rate. These considerations underscore the imprecision in the current definition of DRiPs and the need to investigate to what extent DRiPs result from defects in transcription, splicing, translation, assembly or folding. In the case of STT3B, our data lead us to infer that the high DRiP rate is mainly due to inherent difficulties in protein folding. This is consistent with studies of tyrosinase mutants showing that protein misfolding increases proteasomal degradation and generation of MHC I-associated epitopes (21).

Retrotranslocation is a bottleneck for several ERAD substrates (29). Our data indicate that the limiting step in STT3B processing is retrotranslocation and that this can be alleviated by increasing expression of the immunoproteasome. The latter finding is consistent with the fact that the proteasome located on the cytosolic side of the ER (i.e. mainly the immunoproteasome in jawed vertebrates) is sufficient to retrotranslocate and degrade an ERAD substrate (56). With few exceptions, proteasomal substrates are degraded in a ubiquitin-dependent manner and di-lysine sequences are preferred ubiquitination sites (43, 57). The conserved lysine-rich region at the C-terminus of STT3B contains four di-lysine sequences. We therefore postulate that the STT3B lysine-rich region is a degron because it is a preferred target for ubiquitin conjugation. This would explain why this region is sufficient to enhance generation of MHC-associated OVA peptides and is required for optimal STT3B degradation and processing along the MHC I pathway (Fig. 10). Nevertheless, about two-thirds of MHC–peptide complexes could be generated in the absence of the lysine-rich region (Fig. 10B). This suggests that STT3B may contain other ubiquitination sites and/or that proteasomal degradation of STT3B may be to some extent ubiquitin independent. Indeed, experimental evidence suggests that the sole function of polyubiquitin chains in proteasomal degradation is in tethering substrates to the proteasome (58). Bringing proteins in close proximity to the proteasome while bypassing the ubiquitination step is sufficient to initiate degradation (58). Thus, the close proximity between the immunoproteasome and STT3B (Fig. 4D) could lessen the need for ubiquitination.
Once in the cytoplasm, peptides derived from proteasomal degradation have a very short half-life in vivo. ~7 s for 9-mer peptides (28). More than 99% of peptides are degraded by cytosolic peptidases before they bind TAP (on the cytosolic side of the ER) and thereby enter the MHC I presentation pathway. Thus, the competition for peptides between peptidases and TAP drastically decreases the efficiency of MHC I epitope generation. As a corollary, the probability that a peptide generated by the proteasome will associate with an MHC I molecule should be maximal when the proteasome is located closest to TAP, that is, on the cytosolic face of the ER. Recent data even suggest that proteasomes on the cytosolic side of the ER interact with TAP, thereby supporting the concept of direct targeting of antigenic peptides to the ER via a TAP–proteasome association (59). These data lead us to propose that location of STT3B in the ER membrane is crucial for its contribution to the immunopeptidome because it brings STT3B in close proximity to both the immunoproteasome and TAP. Moreover, preferential degradation by the immunoproteasome, as opposed to the constitutive proteasome, may increase the contribution of STT3B to the immunopeptidome. Indeed, bioinformatic studies predict that the immunoproteasome generates peptides that are better ligands for MHC binding than peptides generated by the constitutive proteasome (60). In contrast with the housekeeping proteasome, the immunoproteasome is found only in vertebrates with an adaptive immune system (gnathostomes) and has co-evolved with the MHC to optimize antigen presentation in gnathostome cells (60, 61).

One fundamental question raised by our work is whether the behavior of STT3B can be extended to other ERAD substrates. In other words, are ERAD substrates a preferential source of MHC I-associated peptides? We anticipate that the answer to this question will be dependent on cell type. First, because the contents in housekeeping proteasome and immunoproteasome are dependent on cell type. Second, because the quantity of ERAD substrates produced varies as a function of the cell’s rate of protein synthesis and secretion (62). Nonetheless, we envision at least two reasons why the contribution of ERAD substrates to the MHC I immunopeptidome might have considerable biologic relevance: (i) central tolerance to self is induced strictly by peptides produced by the immunoproteasome because this is the only proteasome expressed by cells in the thymic medulla (63), and (ii) the immunoproteasome is the dominant type of proteasome found in dendritic cells, the most important antigen-presenting cells (64). Moreover, one could even see the crucial contribution of ER membranes in antigen cross-presentation (65, 66) as a means to use the ERAD machinery to quickly modify the MHC I immunopeptidome.

Seminal studies by other groups strongly suggest that the contribution of a protein to the immunopeptidome is not determined by a single factor. Thus, the extent of protein misfolding, the degradation rate and localization in the cytosol versus the secretory pathway were relevant for some but not all proteins (21, 53, 67, 68). Our work suggests that the prominent imprint of STT3B on the MHC I immunopeptidome results from the interplay of three factors that render STT3B an optimal ERAD substrate: its propensity to misfold, its lysine-rich degron and its location in the ER membrane. Moreover, our analyses show that coupling to STT3B can be used to foster MHC I presentation of a specific peptide. Preliminary work suggests that the same effect can also be achieved with STT3A and with truncated STT3B constructs (data not shown). Given the recent demonstration that a substantial portion of the MHC II immunopeptidome is generated by a proteasome- and TAP-dependent pathway (69), it will be interesting to evaluate whether coupling to STT3B could be used to generate not only MHC I but also MHC II epitopes. This approach could be relevant for application in developing vaccines against selected proteins such as oncoproteins.

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Abbreviations

aa amino acid
DRIP defective ribosomal product
ER endoplasmic reticulum
ERAD endoplasmic reticulum-associated degradation
FBS fetal bovine serum
HA hemagglutinin
MFI mean fluorescence intensity
MTOC microtubule organizing center
NEM N-ethylmaleimide
ORF open reading frame
OVA ovalbumin peptide
PIC protease inhibitor cocktail
PMSE phenylmethylsulfonfluoride
RT room temperature
SIMP source of immunodominant MHC-associated peptides
TBS Tris-buffered saline

References


