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Processing in the Endoplasmic Reticulum Generates an Epitope on the Insulin A Chain that Stimulates Diabetogenic CD8 T Cell Responses

Helen Brosi,2* Michael Reiser,2* Tarvo Rajasalu,† Andreas Syprantis,* Franz Oswald,* Bernhard Otto Boehm,* and Reinhold Schirmbeck3*

RIP-B7.1 mice express the costimulator molecule B7.1 (CD80) on pancreatic β cells and are a well-established model for studying de novo induction of diabetogenic CD8 T cells. Immunization of RIP-B7.1 mice with preproinsulin (ppins)-encoding plasmid DNA efficiently induces experimental autoimmune diabetes (EAD). EAD is associated with an influx of CD8 T cells specific for the K\(^{b}/\)A\(_{12-21}\) epitope into the pancreatic islets and the subsequent destruction of β cells. In this study, we used this model to investigate how ppins-derived Ags are expressed and processed to prime diabetogenic, K\(^{b}/\)A\(_{12-21}\)-specific CD8 T cells. Targeting the K\(^{b}/\)A\(_{12-21}\) epitope, the insulin A chain, or the ppins to the endoplasmic reticulum (ER) (but not to the cytosol and/or nucleus) efficiently elicited K\(^{b}/\)A\(_{12-21}\)-specific CD8 T cell responses. The K\(^{b}/\)A\(_{12-21}\) epitope represents the COOH terminus of the ppins molecule and, hence, did not require COOH-terminal processing before binding its restriction element in the ER. However, K\(^{b}/\)A\(_{12-21}\)-specific CD8 T cells were also induced by COOH-terminally extended ppins-specific polypeptides expressed in the ER, indicating that the epitope position at the COOH terminus is less important for its diabetogenicity than is targeting the Ag to the ER. The K\(^{b}/\)A\(_{12-21}\) epitope had a low avidity for K\(^{b}\) molecules. When epitopes of unrelated Ags were coprimed at the same site of Ag delivery, “strong” K\(^{b}\)-restricted (but not DP\(^{r}\)-restricted) CD8 T cell responses led to the suppression of K\(^{b}/\)A\(_{12-21}\)-specific CD8 T cell priming and reduced EAD. Thus, direct expression and processing of the “weak” K\(^{b}/\)A\(_{12-21}\) epitope in the ER favor priming of autoreactive CD8 T cells. The Journal of Immunology, 2009, 183: 0000 – 0000.

Type 1 diabetes mellitus (T1D) is the result of immunemediated destruction of the insulin-producing β cells in pancreatic islets. T1D in humans is associated with an immune response directed against the β cell-specific Ag, insulin (1). Disease development is triggered by poorly defined factors that finally result in the breakdown of tolerance and activation of autoreactive T cells (1). Animal models have been informative for characterizing the cross-talk between pathogenic CD4 and CD8 T cells with their target organ (i.e., the pancreas); however, the “translation” of these findings to a human T1D therapy has not been successful to date (2, 3).

There is increasing evidence from patients with T1D and from murine models of diabetes that autoreactive CD8 T cells contribute significantly to the development of T1D (1, 4–10). We used RIP-B7.1 mice (11) that express the costimulator molecule B7.1 (CD80) on pancreatic β cells to characterize the specificity and diabetogenic potential of CD8 T cell responses (12–15). Studies have shown that DNA vectors encoding murine preproinsulin-II (ppins) efficiently induced CD8 T cell-dependent experimental autoimmune diabetes (EAD) in RIP-B7.1 (but not B6) mice within 3–4 wk after immunization (15). The EAD was characterized by insulitis, progressive invasion of T cells into the islets, β cell destruction, and hyperglycemia (15). Destruction of pancreatic β cells depended on IFN-γ-producing, ppins-specific CD8 T cells (15). We previously showed that RIP-B7.1 mice deficient in IFN-γ could not induce EAD after immunization with pCI/ppins DNA (15). It has been suggested that IFN-γ may directly mediate β cell destruction and/or facilitate MHC class I-restricted presentation of the diabetogenic epitope(s) to CD8 T cells (16–18). The mechanisms involved in processing diabetogenic epitope(s) from ppins are unknown, and it is not understood how DNA-based immunization with ppins can readily break tolerance and efficiently induce EAD.

In immunized RIP-B7.1 mice, after transfection with ppins-encoding DNA, professional APCs that process preproinsulin to proinsulin (15) and β cells that process ppins to bioactive insulin must present the same antigenic epitope(s) to CD8 T cells to trigger EAD. Ppins is translocated to the endoplasmic reticulum (ER), and it is efficiently processed to proinsulin (pins) by removing the signal peptide (SP). MHC class I molecules display peptides of 8–10 residues from intracellular proteins on the cell surface to CD8 T cells. Most of these peptides are derived from proteins through degradation by proteasomes and are delivered to newly synthesized, ER-resident class I molecules by TAP (19). It is assumed that proteasomes generate the COOH termini of MHC class I-binding
peptides and that ER-associated aminopeptidases trim off NH\textsubscript{2}-terminal extensions (20, 21). Direct ER-associated Ag processing and subsequent MHC class I-loading of antigenic peptides has been described (10, 22–26). For example, some ER-targeting signal sequences contain a CD8 T cell epitope with a final COOH terminus that is released by ER-resident signal peptides (10, 26).

In vitro analyses showed that presentation of these epitopes required neither proteasomes nor TAP (10, 26). This streamlined processing may increase the presentation efficiency of these epitopes (23, 27).

Several epitopes were localized at the extreme COOH terminus of the 24 residue human ppins SP (i.e., ppins\textsubscript{15–24} and ppins\textsubscript{17–24}) (10). CD8 T cells with these specificities were found in patients with T1D, suggesting that they may be directly involved in this disease (10). In the RIP-B7.1 model, ppins-specific DNA immunization efficiently induced insulin A chain (K\textsubscript{\beta}/A\textsubscript{12–21})-specific CD8 T cells (15). Because the COOH terminus of the K\textsubscript{\beta}/A\textsubscript{12–21} epitope corresponds to that of the ppins molecule (15, 28), no further COOH-terminal proteolytic trimming is necessary.

Here we explored the priming conditions for K\textsubscript{\beta}/A\textsubscript{12–21}-specific CD8 T cells in RIP-B7.1 mice. We investigated whether targeting plasmid-encoded ppins, the insulin A chain, or the Kb/A\textsubscript{12–21} epitope to different cellular compartments (ER, cytosol, cytosol/nucleus) facilitated (or did not allow) priming of K\textsubscript{\beta}/A\textsubscript{12–21}-specific CD8 T cells and EAD in immunized RIP-B7.1 mice. This approach was useful for characterizing the in vivo processing requirements of the K\textsubscript{\beta}/A\textsubscript{12–21} epitope.

### Materials and Methods

#### Mice

RIP-B7.1 mice were backcrossed for >15 generations to the C57BL/6 (H-2\textsuperscript{b}) background as described previously (11). Mice were bred and maintained under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Male and female mice were used in the experiments at 6–8 wk of age.

#### Construction of expression plasmids

The antigenic sequences were synthesized by GeneArt and cloned into the pCI vector (Promega, catalog no. E1731) with the Nehl and NotI restriction sites. Batches of DNA were produced in Escherichia coli (PlasmidFactory). For construction of the pCI/rd fluorescent protein (RFP)-ER vector, the DNA sequence for an ER targeting signal peptide (METDTLLLWVLWVPGSTGD) derived from the murine IgG chain was attached to the NH\textsubscript{2} terminus DNA sequence of a RFP (eqF6011) (29) by PCR. The resulting RFP-encoding sequence was further modified by using PCR to attach an ER retention signal sequence (SEKDEL, 30) to the COOH terminus.

#### Characterization of Ag expression

HEK cells were transiently transfected with the indicated plasmid DNA using the calcium phosphate method. Cells were either labeled with 35S-methionine/cysteine between 36 and 48 h after transfection or directly lysed 48 h after transfection. Extracts were cleared by centrifugation and precipitated with either rabbit insulin Ab (Santa Cruz Biotechnology, catalog no. sc-9168) or anti T-Ag mAb (to immunoprecipitate T\textsubscript{77}o rc T\textsubscript{272} from Western blot analyses). The secondary Ab, FITC-tabeled anti-guinea pig IgG (Sigma-Aldrich, catalog no. F-6261) (1/100), was used to detect insulin-bound immune complexes.

### Determination of blood glucose levels

Diabetes was diagnosed when two consecutive blood glucose values exceeded 250 mg/dl (13.8 mmol/L; Disetronic Freestyle).

#### Histology

Pancreatic cryosections (3 \textmu m thick) were pretreated with blocking goat serum (Zymed Laboratories, catalog no. 50-1972) and incubated with a primary guinea pig Ab to insulin (Dako, catalog no. A0564) and a PE anti-CD8 Ab (clone 53-6.7; BD Pharmingen). The secondary Ab, FITC-tabeled anti-guinea pig IgG (Sigma-Aldrich, catalog no. F-6261) (1/100), was used to detect insulin-bound immune complexes.

### Immunofluorescence

Immunofluorescence

HeLa cells were grown in twin chambers (Nunc, catalog no. 155380) in 2 ml of medium (Invitrogen, catalog no. 31885) supplemented with 10% FCS. Chambers were pretreated with fibronectin (Roche, cat. no. 10838039001) to enhance attachment of cells. Where indicated, cells were cotransfected with GFP- and RFP-encoding plasmids with the Nanofect transfection reagent (PAA, catalog no. Q051005). Images of living cells were directly acquired 24 h after transfection with a fluorescence microscope (IX71; Olympus) equipped with a digital camera (C4742; Hamamatsu), a 100-W mercury lamp (HBO 103W/2; Osram), and the following filter sets: GFP, excitation HQ470/40, emission HQ525/50; RFP, excitation HQ545/30, emission HQ610/75 (AHF Analysentechnik). For confocal microscopy, cells were transfected with the indicated GFP-expressing plasmids, fixed with 4% PFA, and permeabilized with 0.2% Triton X-100. Fish-skin gelatin (0.2%) was used as blocking reagent. Cells were stained for calnexin with rabbit anti-calnexin (Santa Cruz Biotechnology, catalog no. sc-11397) and TRITC-conjugated anti-rabbit Ab (Abcam, catalog no. ab50598). The colocalization of GFP-tagged fusion proteins was analyzed directly or after enhancement with anti-rabbit anti-GFP and FITC-conjugated anti-rabbit Ab by excitation with a 488-nm laser. Images were acquired using a spinning disc confocal microscope.

#### Preparation of pancreatic CD8 T cells

Pancreata were perfused in situ with collagenase D dissolved in HBSS (1 mg/ml) (Roche, catalog no. 11213865001). Thereafter, pancreata were removed, digested with collagenase D for 8 min at 37°C, and washed twice with cold HBSS supplemented with 10% FCS. Pancreatic CD8 T cells were purified with Histopaque-1077 (Sigma-Aldrich, catalog no. 10771) by centrifugation for 15 min at 2400 rpm.

#### Determination of specific CD8 T cell frequencies

To detect ppins-specific CD8 T cell responses, we used a peptide variant with a K\textsubscript{\beta}/A\textsubscript{12–21} epitope that had a substitution of N to A at position 21 (SLYQLENYCA). This epitope variant facilitated in vitro expansion of primed CD8 T cells (data not shown). Briefly, pancreatic CD8 T cells (1 \times 10\textsuperscript{7}/100 \mu l) were incubated overnight with 2–20 \mu g/ml of the indicated peptides in the presence of brefeldin A. Cells were harvested, washed, and surface stained with allophycocyanin-conjugated anti-CD8 Ab (BD Biosciences, catalog no. 554411) for 30 min at room temperature, and washed twice in permeabilization buffer. Frequencies of IFN-\gamma-producing CD8 T cells were determined by flow cytometry (FCM) analyses. Hepatitis B surface Ag (HBsAg)-specific CD8 T cells were detected with tetramer staining as described previously (32). Where indicated, the values were analyzed with GraphPad Prism software, version 4.0 (GraphPad Software). The statistical significance of differences in the mean CD8 T cell frequencies between groups was determined with an unpaired Student’s t test. A value of p < 0.05 was considered significant.

### Results

#### Immunization of RIP-B7.1 mice with insulin-encoding plasmids induced EAD

RIP-B7.1 mice (on the C57BL/6 background) express the costimulatory molecule B7.1 (CD80) selectively in pancreatic \beta cells. A single i.m. injection of pCI/ppins plasmid DNA encoding murine preproinsulin-II (ppins) (Fig. 1A) efficiently induced hyperglycemia in RIP-B7.1 mice (Fig. 1B) (15) with an onset of EAD after...
3–4 wk (Fig. 1C). RIP-B7.1 mice with control vector injections did not develop EAD (Fig. 1B). The cumulative diabetes incidence was >95% at 4 wk postimmunization (Fig. 1C; group 1). EAD was efficiently induced by pCl/ppins DNA in MHC class II-deficient (Aα−/−) RIP-B7.1 mice (RIP-B7.1/Aα−/−) with no conventional CD4 T cells (Fig. 1C, group 2) and in RIP-B7.1 mice that were acutely depleted of CD4 T cells with mAb YTS-191 (15). Thus, EAD was induced by a CD4 T cell-independent, diabetogenic CD8 T cell response to ppins in this model.

Pancreas-infiltrating CD8 T cells isolated from immunized, diabetic RIP-B7.1 mice specifically recognized the Kβ-restricted A12–21 epitope of ppins (Fig. 1D) (15, 28). Ex vivo stimulation of ppins-primed CD8 T cells with either Kβ-expressing P1/Kβ transfectants pulsed with the antigenic A12–21 peptide or stable ppins-expressing RBL5/ppins (H-2b) transfectants revealed a CD8 T cell subset with specifically inducible IFN-γ expression (Fig. 1D).

The development of EAD in pCl/ppins-immunized RIP-B7.1 mice is characterized by CD8 T cell infiltration into pancreatic islets (destructive insulitis) and insulin deficiency. We detected CD8 T cell infiltration and destruction of islet cells in immunized RIP-B7.1 mice by immunohistochemistry (Fig. 2A). At day 2 postimmunization, the pancreatic islets of pCl/ppins-immunized,
healthy RIP-B7.1 mice contained barely detectable amounts of CD8 T cells, and insulin expression was intact (Fig. 2A). By day 12 postimmunization, the animals were normoglycemic, but insulin expression was reduced in the islets, and CD8 T cell infiltration had increased (Fig. 2A). At 24 days postimmunization, extensive insulitis, CD8 T cell infiltration, and hyperglycemia were accompanied by reduced insulin expression (Fig. 2A). As the disease progressed, we detected increased K\(^{b}/A_{12–21}\)-specific IFN-\(\gamma\) CD8 T cell infiltration in the pancreas (Fig. 2B). In severe disease, 10–16% of all intrapancreatic IFN-\(\gamma\)-producing CD8 T cells were K\(^{b}/A_{12–21}\)-specific (Fig. 2B). Hence, the development of EAD was accompanied by increasing infiltrations of K\(^{b}/A_{12–21}\)-specific CD8 T cells into the target tissue.

**Expression of ppins in the ER facilitated priming of K\(^{b}/A_{12–21}\)-specific CD8 T cells**

When the entire ppins-encoding gene was introduced into nonpancreatic cells (as in DNA immunization, or transient transfection of HEK cells), ppins was expressed and translated into the ER, where the signal peptide was removed to generate pins (15) (Fig. 3A). Using a ppins mutant with an Ab-binding hemagglutinin domain (HA-tag) fused COOH terminally in frame to the insulin A chain (pCI/ppins-HA), we have not detected further downstream processing of pins to the insulin A chain or secretion of ppins-specific intermediates (15). ER-associated pins was thus expected to be the major substrate for generating diabetogenic epitopes in nonpancreatic cells.

We tested whether localization of pins in the cytosol could lead to induction of K\(^{b}/A_{12–21}\)-specific CD8 T cells and EAD in RIP-B7.1 mice. Toward this end, we expressed pins in the cytosol by removing the insulin-specific signal peptide (pCI/pins). This pins protein was not detectable in transiently transfected cells (Fig. 3B, lane c), but addition of proteasome inhibitors (a mixture of eoxymycin and lactacystin) efficiently restored cytosolic pins levels within 4 h (Fig. 3B, lane d). This showed that quantitative, proteasome-mediated degradation of cytosolic pins resulted in a high turnover of this Ag. In contrast, expression of ppins-derived, ER-associated pins was not modulated by proteasome inhibitors (Fig. 3B, lanes a and b). Thus, proteasomes degrade cytosolic, but not ER-associated, pins.

Injection of the pCI/pins vector DNA inefficiently induced late EAD in RIP-B7.1 mice (Fig. 3C, group 2). K\(^{b}/A_{12–21}\)-specific CD8 T cells were barely detectable in these diabetic mice (Fig. 3D, group 2). These findings indicate that induction of diabetogenic T cell responses are different in pCI/ppins- and pCI/pins-immunized RIP-B7.1 mice, and they confirm previous findings reported for the NOD mouse system (33). Thus, immunization of RIP-B7.1 mice with the pCI/pins vector may lead to EAD by inducing CD8 T cell responses that have specificities other than K\(^{b}/A_{12–21}\). In support of this hypothesis, we showed that a mutant ppins with a deletion of the A\(_{12–21}\) sequence (pCI/ppinsA\(_{12–21}\)) induced CD8 T cell-mediated EAD in RIP-B7.1 mice (Fig. 3, C and D, group 3). Diabetogenic T cell specificities primed by immunization with pCI/pins or pCI/ppinsA\(_{12–21}\) are currently not mapped.

**K\(^{b}/A_{12–21}\)-monospecific CD8 T cells efficiently induced EAD in RIP-B7.1 mice**

Proteins can be directed to the ER by fusing them with a NH\(_{2}\)-terminal signal sequence (METHDLLLLWLLLLWPGSTGD) derived from the murine Ig\(\kappa\) chain (34). We constructed two vectors: the pCI/L-A\(_{12–21}\) vector that encodes only the insulin K\(^{b}/A_{12–21}\) sequence, and the pCI/L-A\(_{12–21}\) vector that encodes the insulin K\(^{b}/A_{12–21}\) sequence and the Ig\(\kappa\) leader (L) sequence (Fig. 4A). Injection of pCI/L-A\(_{12–21}\) (with the ER-targeting sequence), but not pCI/L-A\(_{12–21}\), induced K\(^{b}/A_{12–21}\)-specific CD8 T cells and EAD in RIP-B7.1 mice (Fig. 4B, groups 1 and 2). Similarly, injection of the insulin A chain encoding pCI/L-A\(_{12–21}\), but not pCI/A\(_{12–21}\) vector (Fig. 4B), efficiently induced EAD in RIP-B7.1 mice (Fig. 4B, groups 3 and 4). This showed that CD8 T cells (monospecific for the insulin K\(^{b}/A_{12–21}\) epitope induced EAD in RIP-B7.1 mice. Thus, induction of diabetogenic, K\(^{b}/A_{12–21}\)-specific CD8 T cells was observed when the antigenic K\(^{b}/A_{12–21}\) peptide or the A chain was expressed in the ER.

**Stable expression of the ppins in the ER but not in the cytosol/nucleus induced diabetogenic CD8 T cells in immunized RIP-B7.1 mice**

We used a heat shock protein (Hsp)-facilitated expression system to target the ppins or the insulin A chain to the cytosol (31, 35) to test whether it could induce priming of K\(^{b}/A_{12–21}\)-specific, diabetogenic CD8 T cells. The Hsp-mediated expression system is based...
injections of T77-ppins or pCI/cT272-A DNA, they did not develop
when RIP-B7.1 mice were immunized with either single or repeated
ulators of the innate and adaptive immune system (37). However,
high steady-state levels in transfected cell lines. Hsps are potent stim-

The positions of the Hsp-binding cT272 and T77 fragments, the insulin A
C
terminus, were determined.

transfection, cells were labeled for 12 h with35S-methionine, immunopre-

fected with pCI/T77-ppins (lane 1) or pCI/cT272-A (lane 2). At 36 h after
transfection, cells were labeled for 12 h with 35S-methionine, immunopre-
cepted with an anti-T mAb, and analyzed by SDS-PAGE and gel fluo-

FIGURE 4. Induction of diabetogenic CD8 T cell responses by ppins
Ags expressed in the ER. A, Schematic presentation of the expression con-
structs. The positions of the entire insulin A1–21 chain, the A12–21 fragment,
and the murine Igκ-leader sequence are indicated. A start methionine was
cloned in front of the A1–21 and A12–21 sequences encoded by the pCI/
A1–21 and pCI/A12–21 vectors, respectively. B, RIP-B7.1 mice were immu-
nized with pCI/A1–21 (n = 5; group 1), pCI/L-A1–21 (n = 5; group 2),
pCI/A12–21 (n = 5; group 3), pCI/L-A1–21 (n = 5; group 4), or pCI/T77-
ppins (n = 8; group 5) and cumulative diabetes incidences (%) were de-
determined. C, Schematic presentation of Hsp-binding expression constructs.
The positions of the Hsp-binding cT272 and T77 fragments, the insulin A
chain, and the ppins are indicated. D, HEK cells were transiently trans-
fected with pCI/GFP-ppins or pCI/ppins-GFP-ER. Twenty-

terminal SEKDEL ER-retention sequence). Twenty-four hours after trans-
fection, cells were directly supplied to live-imaging immunofluorescence
microscopy using an Olympus IX71 microscope. C, HeLa cells were tran-
siently transfected with pCI/GFP-ppins or pCI/ppins-GFP-ER. Twenty-
four hours after transfection, cells were fixed with 4% PFA, permeabilized
with 0.2% Triton X-100, stained with anti-GFP/FITC and anti-calnexin/
TRITC Abs, and analyzed by confocal microscopy. Magnification, ×1000.
Notably, the anti-GFP staining was necessary in these analyses because the
fluorescent GFP signal became weak in fixed cells.

FIGURE 5. Expression of GFP/ppins fusion Ags. A, Schematic presen-
tation of expression constructs. The positions of the GFP (eGFP), the ppins
(SP-B-C-A), the ER-retention sequence (SEKDEL), and of the Kb/A12–21
epitope are indicated. B, HeLa cells were transiently cotransfected with a
RFP-ER-tagging vector (that expressed a red fluorescent protein tagged
in the ER) and either pCI/GFP-ppins DNA (that encoded eGFP fused with
the COOH-terminal ppins sequence) or a ppins-GFP-ER-fusing vector
(that encoded eGFP with the NH2-terminal ppins sequence and the COOH-
terminal SEKDEL ER-retention sequence). Twenty-four hours after trans-
fection, cells were directly supplied to live-imaging immunofluorescence
microscopy using an Olympus IX71 microscope. C, HeLa cells were tran-
siently transfected with pCI/GFP-ppins or pCI/ppins-GFP-ER. Twenty-
four hours after transfection, cells were fixed with 4% PFA, permeabilized
with 0.2% Triton X-100, stained with anti-GFP/FITC and anti-calnexin/
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Notably, the anti-GFP staining was necessary in these analyses because the
fluorescent GFP signal became weak in fixed cells.

on NH2-terminal, DnaJ-like sequences of cytosolic SV40 large
T-Ag (cT272; T77) that efficiently capture and noncovalently bind the
constitutively expressed, cytosolic Hsp73 protein (31, 35). Reporter
Ags expressed in the cytosol did not enter the ER, and they
were associated with Hsp73 but not ER-resident Hsps (Ref. 36 and

and ppins in the ER is essential for priming diabetogenic Kb/A12–21
epitope.

We further used a GFP (eGFP)-based reporter system to exclude
(or localize) the ppins or the insulin A chain from (to) the ER. GFP
was fused to either the NH2 (pCI/GFP-ppins) or the COOH ter-
minus (pCI/ppins-GFP-ER) of the ppins (Fig. 5A). In the later
construct, the ppins-GFP sequence was further fused COOH-ter-
minally with the ER retention signal (SEKDEL) to retard the
ppins-GFP in the ER (30). The receptor for the SEKDEL retention
signal resides in an early Golgi compartment and function through a
recycling process that retrieves ER proteins back from the Golgi
apparatus to the ER (30). The subcellular localization of the insu-
lin/GFP fusion proteins was analyzed in HeLa cells transiently
transfected with the respective vectors and an ER-tagged RFP plasmid, pCI/RFP-ER (29). Live-imaging immunofluorescence microscopy showed that the GFP-ppins fusion protein was localized in the nucleus and cytosol, but not in the ER (Fig. 5B). In contrast, the signal peptide of ppins directed the ppins-GFP-ER protein into the ER, and this fusion protein was localized to the ER, but was not observed in the nucleus or cytosol (Fig. 5B). We confirmed the intracellular distribution of ppins-GFP-ER and GFP-ppins proteins by confocal microscopy (Fig. 5C). HeLa cells transfected with pCI/GFP-ppins or pCI/ppins-GFP-ER DNA were stained for calnexin. The ppins-GFP-ER but not GFP-ppins colocalized with the ER-resident calnexin (Fig. 5C). Insulin- and GFP-specific Western blot analyses showed that GFP-ppins and ppins-GFP-ER fusion proteins were efficiently expressed in transiently transfected HEK cells, and no expression of intermediate products was detectable (Fig. 6, A and B). Notably, the expression levels of insulin/GFP fusion Ags were >200-fold higher than of the ppins expressed by the pCI/ppins vector (Fig. 6A, groups 1, 3, and 4).

We immunized RIP-B7.1 mice with the respective vectors. The pCI/ppins-GFP-ER but not the pCI/GFP-ppins DNA induced EAD in RIP-B7.1 mice (Fig. 6C, groups 1 and 2). High frequencies of K\textsuperscript{b}/A\textsubscript{12–21}-specific IFN-\gamma\textsuperscript{+} CD8 T cells were detectable in pCI/ppins-GFP-ER-immunized, diabetic RIP-B7.1 mice (Fig. 6C, group 2) but not in healthy, pCI/GFP-ppins-immunized RIP-B7.1 mice (Fig. 6C, group 1). Repeated injections of pCI/GFP-ppins or a pCI/GFP-A vector (expressing a cytotoxic/nucleosolic GFP/insulin A chain fusion protein) did not induce EAD in RIP-B7.1 mice (data not shown). This confirmed that this T cell specificity was efficiently primed by processing ER-associated (but not cytosolic or nuclear) Ags. Furthermore, considering that the ppins-GFP-ER construct encodes the ppins (and thus the K\textsuperscript{b}/A\textsubscript{12–21} epitope) COOH terminally flanked by the GFP-SEKDEL (Fig. 5A), these findings suggested that the epitope position at the COOH terminus is less important for its diabetogenicity than is targeting the Ag to the ER.

**Copriming CD8 T cells with K\textsuperscript{b}-restricted viral epitopes prevents priming of diabetogenic CD8 T cells**

The K\textsuperscript{b}/A\textsubscript{12–21} epitope inefficiently stabilized K\textsuperscript{b} molecules on the surface of TAP-deficient RMA-S cells. This suggested that the K\textsuperscript{b}/A\textsubscript{12–21} epitope has only low avidity for K\textsuperscript{b} (38) (Fig. 7A). To determine whether priming of K\textsuperscript{b}/A\textsubscript{12–21}-specific, autoreactive CD8 T cells might be limited by “strong” K\textsuperscript{b}-restricted CD8 T cell responses to epitopes of unrelated Ags (2, 3), we established in vivo immune competition experiments.

The pCI/S plasmid was used to express the secreted small hepatitis B surface Ag (HBsAg or S). This Ag contains a well-defined Db- but no Kb-restricted epitopes (41, 42) or a mixture of pCI/ppins and pCI/S plasmids depended on the cellular expression of the HBsAg (43). Notably, the expression levels of HBsAg/S were >200-fold higher than of the ppins expressed by the pCI/ppins vector (Fig. 6A, groups 1, 3, and 4).

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We immunized RIP-B7.1 mice with the respective vectors. The pCI/ppins-GFP-ER but not the pCI/GFP-ppins DNA induced EAD in RIP-B7.1 mice (Fig. 6C, groups 1 and 2). High frequencies of K\textsuperscript{b}/A\textsubscript{12–21}-specific IFN-\gamma\textsuperscript{+} CD8 T cells were detectable in pCI/ppins-GFP-ER-immunized, diabetic RIP-B7.1 mice (Fig. 6C, group 2) but not in healthy, pCI/GFP-ppins-immunized RIP-B7.1 mice (Fig. 6C, group 1). Repeated injections of pCI/GFP-ppins or a pCI/GFP-A vector (expressing a cytotoxic/nucleosolic GFP/insulin A chain fusion protein) did not induce EAD in RIP-B7.1 mice (data not shown). This confirmed that this T cell specificity was efficiently primed by processing ER-associated (but not cytosolic or nuclear) Ags. Furthermore, considering that the ppins-GFP-ER construct encodes the ppins (and thus the K\textsuperscript{b}/A\textsubscript{12–21} epitope) COOH terminally flanked by the GFP-SEKDEL (Fig. 5A), these findings suggested that the epitope position at the COOH terminus is less important for its diabetogenicity than is targeting the Ag to the ER.

**Copriming CD8 T cells with K\textsuperscript{b}-restricted viral epitopes prevents priming of diabetogenic CD8 T cells**

The K\textsuperscript{b}/A\textsubscript{12–21} epitope inefficiently stabilized K\textsuperscript{b} molecules on the surface of TAP-deficient RMA-S cells. This suggested that the K\textsuperscript{b}/A\textsubscript{12–21} epitope has only low avidity for K\textsuperscript{b} (38) (Fig. 7A). To determine whether priming of K\textsuperscript{b}/A\textsubscript{12–21}-specific, autoreactive CD8 T cells might be limited by “strong” K\textsuperscript{b}-restricted CD8 T cell responses to epitopes of unrelated Ags (2, 3), we established in vivo immune competition experiments.

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We found that small antigenic peptides (i.e., the A12–21 epitope and corresponding cytosolic peptides (pCI/A12–21; pCI/A1–21), could efficiently elicit Kb/A12–21-specific CD8 T cells and EAD in RIP-B7.1 mice. The ER-targeted A12–21 peptide is released by signal peptidases in the ER and can bind to nascent MHC class I molecules in the ER without further processing. We assumed the L-A12–21 fusion peptide was expressed at high enough levels that sufficient amounts of the Kb/A12–21 epitope were released within the ER (by signal peptidases) to load a critical number of the nascent Kb molecules to prime CD8 T cell responses (22, 23). These peptide concentrations may not be reached when the A12–21 peptide was expressed in the cytosol and must compete with the bulk of high-affinity, antigenic peptides (generated in the conventional Ag presentation pathway by proteosomal degradation) for TAP-dependent transport into the ER (43). Processing and loading of the entire, ER-targeted insulin A chain (L-A12–21) to the Kb molecules may also proceed in the ER. When epitopes with a final COOH terminus are bound by MHC class I molecules in the ER, the epitopes are trimmed by ER-resident aminopeptidases (10, 20, 21).

Targeting the vector-encoded insulin A chain or the ppins into different cellular compartments (cytosol, cytosol/nucleus) by fusing it to Hsp73-binding T-Ag fragments or COOH terminally to GFP significantly enhanced expression levels of these autoantigens, but did not induce Kb/A12–21-specific CD8 T cell responses or EAD in immunized RIP-B7.1 mice. The immunogenicity of DNA vaccines that encode Hsp73-captured chimeric Ags was often enhanced compared with the parental Ags (37). The Hsp73-mediated stimulus of the immune system did not facilitate priming of Kb/A12–21-specific CD8 T cells. Thus, the diabetogenic epitope(s) cannot be (or are inefficiently) presented from ppins-Ags that are excluded from the ER. However, a highly unstable, cytosolic pins Ag (encoded by the pCI/pins vector) induced low levels of Kb/A12–21-specific CD8 T cells and triggered late EAD in RIP-B7.1 mice. This indicates that this epitope could be marginally present in an ER-independent mechanism in vivo. We assumed that additional T cell specificities to yet unknown epitopes-caused EAD in pCI/pins immunized RIP-B7.1 mice. Similarly, Kb/A12–21-independent EAD could be induced in RIP-B7.1 mice with a pCI/ppins-L-A12–21 vector. This construct encoded an ER-associated pins without the Kb/A12–21 sequence and induced CD8 T cell-mediated EAD. Notably, these pins-specific, diabetogenic T cell specificities were not induced by the Ags (i.e., T77/ppins, GFP-ppins) expressing the pins fusion proteins in the cytosol/nucleus. Thus, expression of pins Ags in the ER played a general role in priming different pins-specific CD8 T cell responses.

The Kb/A12–21 epitope could also be generated from different, COOH terminally extended polypeptides in the ER. We showed that the ppins-GFP-ER fusion Ag (encoding the ppins and thus the Kb/A12–21 epitope COOH terminally flanked by the GFP) or a ppins-HA Ag (encoding the ppins COOH terminally flanked by a nine-residue YPYDVPDYA hemagglutinin domain) (15) efficiently induced Kb/A12–21-specific CD8 T cells and EAD in immunized RIP-B7.1 mice (Fig. 6 and data not shown). Similarly, a pCI/L-A12–21-ER vector encoding the L-A12–21 sequence COOH terminally fused with the six-residue ER-retention signal SEKDEL efficiently induced (mono)specific CD8 T cells and EAD in immunized RIP-B7.1 mice (data not shown). These ER-resident Ags require further proteolytic removal of the COOH-terminal sequences to generate the final COOH terminus of the Kb/A12–21 epitope. However, we do not know how and where these Ags are processed to remove the COOH-terminal sequences. COOH-terminal trimming of epitopes has been described for cytosolic pro teaseoms (20, 21) but ER-resident peptidase subsets in these processes are yet not well understood (24, 25).

In nonpancreatic cells, pins is expressed and translocated into the ER where the signal peptide is removed to generate pins. Farther downstream, processing of pins to insulin is not detectable as previously reported (15). ER-associated pins (but not other downstream intermediates or secreted insulin) is thus expected to be a
major substrate for generating diabetogenic epitopes in nonpancreatic APCs (15). We generated a ppins mutant that cannot be processed to bioactive insulin. The two pairs of dibasic signal residues (RRK \(_{55-56}\) and KRK \(_{88-89}\)), dissecting the B and A chains from the C peptide, are essential enzymatic processing sites to release insulin B and A chains. Alanine substitutions of these signal residues were thus expected to abolish the natural processing of ppins to insulin. A mutant pCI/ppins-RKmut vector expressed comparable levels of ppins in transiently transfected HEK cells as pCI/ppins DNA, and efficiently induced EAD and K\(^{b}/A_{12–21}\)-specific CD8 T cells (data not shown). We also found that it is not essential for ppins to maintain a precise conformation in the ER to prime this diabetogenic T cell response. The ppins contains six cysteines in the B and A chains. Substitution of five of the cysteines (all except the cysteine located in the K\(^{b}/A_{12–21}\) epitope) with alanine in a pCI/ \(_{mut}\) vector efficiently elicited K\(^{b}/A_{12–21}\)-specific CD8 T cells and EAD in immunized RIP-B7.1 mice (data not shown). Thus, we suggest that the expression of Ags in the ER, rather than Ag conformation or its natural processing to insulin, are required for efficient priming of diabetogenic CD8 T cell responses. Moreover, synthetic A chain peptides (A\(_{12–21}\cdot A_{1–21}\) co-delivered with IFA and/or different Th1-stimulating adjuvant formulations (e.g., CpG containing oligodeoxynucleotides) did not induce EAD in RIP-B7.1 mice (data not shown). This suggested that the K\(^{b}/A_{12–21}\) epitope was inefficiently processed from “exogenous” Ags. Long CD8 T cell epitopes have been described (44), but the K\(^{b}\)-restricted 10-mer A\(_{12–21}\) epitope of insulin contains only an auxiliary anchor motif at position Y3 (45). Specific ex vivo restimulation of primed CD8 T cells requires high doses of the antigenic peptide. Direct biochemical binding assays indicated that this peptide had a very low avidity for K\(^{b}\). This was confirmed when the antigenic A\(_{12–21}\) peptide did not stabilize surface K\(^{b}\) class I molecules on the surface of TAP-deficient RMA-S cells, a widely used assay to measure the avidity of an antigenic peptide for its restriction element. Despite extensive efforts, we have not been able to construct tetramers or pentamers of the K\(^{b}/A_{12–21}\) peptide. Hence, different independent data indicate that we are dealing with a “weak” epitope. The weak classification of the K\(^{b}/A_{12–21}\) epitope was further supported by immune-competition experiments. Copriming of K\(^{b}\) (but not D\(^{b}\)) restricted, HBsAg-specific CD8 T cells at the same site of injection suppressed induction of the ppins-specific, pathogenic CD8 T cell response. Direct loading of the A\(_{12–21}\) epitope on newly synthesized K\(^{b}\)-molecules in the ER may be an essential step for enabling presentation of this weak epitope to diabetogenic CD8 T cells.

It is not known why DNA-based immunization with ppins or insulin A chain Ags can readily break tolerance and efficiently induce EAD in RIP-B7.1 mice. It is assumed that T cells with low avidity for a tissue-restricted Ag can evade central and peripheral tolerance regulation and cause autoimmunity (46, 47). This may also be the case for K\(^{b}/A_{12–21}\)-specific CD8 T cells. These T cells may escape negative selection in the thymus or regulatory T cell control because of being too weak for recognition. K\(^{b}/A_{12–21}\)-specific CD8 T cells and EAD were induced in RIP-B7.1 mice by injection of high doses (50–100 \(\mu\)g DNA) of ppins-encoding vectors. This confirmed that plasmid DNA immunization potently stimulates CD8 T cell responses (48). DNA immunization induces Th1-biased immune responses to most Ags. In contrast, ballist DNA immunization of low amounts (1–2 \(\mu\)g/mouse) of plasmid DNA with the gene gun facilitated Th2-biased immunity (48) and failed to induce EAD in RIP-B7.1 mice even after repeated boost immunizations (data not shown) (49). We thus conclude that induction of autoreactive CD8 T cell responses requires costimulation of the Th1-biased immune system. Alternatively, plasmid DNA-induced innate immune responses may directly facilitate \(\beta\) cell susceptibility to T cell-mediated destruction (50). This is unlikely because K\(^{b}/A_{12–21}\)-monospecific CD8 T cells isolated from diabetic RIP-B7.1 mice immunized with pCI/A\(_{12–21}\) DNA efficiently triggered EAD after adoptive cell transfer into RIP-B7.1 hosts (data not shown) (49). Thus, DNA immunization does not directly influence presentation of the K\(^{b}/A_{12–21}\) epitope by pancreatic \(\beta\) cells.

Mice express two nonallelic insulin-I- and -II genes (51). Murine ppins-I and ppins-II are located on different chromosomes. Murine ppins-I differs from ppins-II by 13 aa residues or 12%. These variant amino acids are found predominantly in the signal peptide and the C peptide of ppins. The insulin A chains (and the A\(_{12–21}\) epitope) are identical in ppins-I and ppins-II. Both ppins proteins are expressed in pancreatic \(\beta\) cells, but only ppins-II is expressed in the thymus. A ppins-II deficiency in NOD mice (4) accelerated progression of diabetes development (52, 53), but a ppins-I deficiency prevented diabetes in most NOD mice (54). This implies that ppins-II expression in the thymus plays an essential role in the negative selection of autoreactive T cells, and that ppins-I is the primary autoantigen that induces diabetes in this mouse model. The different effects of ppins-I and ppins-II DNA vaccines in diabetes development in NOD mice (33) are not observed in the ppins-induced EAD in the RIP-B7.1 mouse model. Both, ppins-I and ppins-II encoding vectors induced EAD in RIP-B7.1 mice with comparable kinetics and incidence (15). This may indicate that different pathogenic T cell responses are primed in NOD and RIP-B7.1 mice. At least, NOD mice express D\(^{b}\) and K\(^{b}\)-MHC class I molecules (4, 5) and do not elicit K\(^{b}/A_{12–21}\)-specific CD8 T cell responses.

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Disclosures

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