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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 Spyrantis,* 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 Kβ/A12–21 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, Kβ/A12–21-specific CD8 T cells. Targeting the Kβ/A12–21 epitope, the insulin A chain, or the ppins to the endoplasmic reticulum (ER) (but not to the cytosol and/or nucleus) efficiently elicited Kβ/A12–21-specific CD8 T cell responses. The Kβ/A12–21 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, Kβ/A12–21-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 Kβ/A12–21 epitope had a low avidity for Kβ molecules. When epitopes of unrelated Ags were coprimed at the same site T cell priming and reduced EAD. Thus, direct expression and processing of the “weak” Kβ/A12–21 epitope in the ER favor priming of autoreactive CD8 T cells. The Journal of Immunology, 2009, 183: 7187–7195.

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4 Abbreviations used in this paper: T1D, type 1 diabetes mellitus; EAD, experimental autoimmune diabetes; ER, endoplasmic reticulum; FCM, flow cytometry; HBsAg, hepatitis B surface Ag; Hsp, heat shock protein; pms, preoinsulin; ppins, preproinsulin; RIP, rat insulin promoter; RFP, red fluorescent protein; SP, signal peptide.

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peptides and that ER-associated aminopeptidases trim off NH2-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 process may increase the presentation efficiency of these epitopes (23, 27). Skowera et al. identified two HLA-A*0201-restricted epitopes that were localized at the extreme COOH terminus of the 24 residue human ppins SP (i.e., ppins15–24 and ppins17–24) (10).

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**Materials and Methods**

**Mice**

RIP-B7.1 mice were backcrossed for >15 generations to the C57BL/6 (H-2b) background as described previously (11). Mice were 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 Nhel and Not restriction sites. Batches of DNA were produced in Escherichia coli (PlasmidFactory). For construction of the pcI/der fluorescent protein (RFP)-ER vector, the DNA sequence for an ER targeting signal peptide (METDTLQDWLWHPGSTDG) derived from the murine Igk chain was attached to the NH2 terminus DNA sequence of a RFP (eqF661l) (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 DNAs 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 T77 or T272–217 specific fusion proteins) and protein A-Sepharose. Precipitates were processed for SDS-PAGE (10–15%) and subsequent gel fluorography or for Western blot analyses. Alternatively, nonlabeled cells were directly lysed with 1.5% SDS, 5% mercaptoethanol, and 20 mM Tris–hydrochloride (pH 8.0) and processed for SDS-PAGE (total cell extracts). Gels were processed for Western blot analyses by probing with rabbit anti-insulin (Santa Cruz Biotechnology, catalog no. sc-9168) or rabbit anti-GFP (Acris, catalog no. AB290) Abs, as described previously (31).

**Immunization of mice**

Intramuscular DNA immunization was achieved by injecting 75–100 μg of plasmid DNA dissolved in PBS into both tibialis anterior muscles.

**Determination of specific CD8 T cell frequencies**

To detect ppins-specific CD8 T cell responses, we used a peptide variant with a K/C/A12–21 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 × 105/100 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. 718619) for 30 min at room temperature, and washed twice in permeabilizing buffer (BD Biosciences, catalog no. 554411) for 30 min at room temperature, and washed twice in permeabilization buffer. Frequencies of IFN-γ+ 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 constitutive molecule B7.1 (CD80) selectively in pancreatic β cells. A single i.m. injection of pCI/ppins plasmid DNA encoding murine propreoinsulin-II (ppins) (Fig. 1A) efficiently induced hyperglycemia in RIP-B7.1 mice (Fig. 1B) (15) with an onset of EAD after...
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 pCI/ppins DNA in MHC class II-deficient (Aα−/−) RIP-B7.1 mice (RIP-B7.1/MHC-II−/−) 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 Kα-restricted A12–21 epitope of ppins (Fig. 1D) (15, 28). Ex vivo stimulation of ppins-primed CD8 T cells with either Kα-expressing P1/Kα 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 pCI/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 pCI/ppins-immunized, - A12–21 epitope of ppins (Fig. 1D) (15, 28). Ex vivo stimulation of ppins-primed CD8 T cells with either Kα-expressing P1/Kα 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).

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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-\(\gamma\) 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 translocated 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 pins-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 epoxymycin 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/ppins\(_{A_{12–21}}\)) 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/ppins\(_{A_{12–21}}\) 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 (MELYDLLWLLLWPGGSTGD) derived from the murine Ig \(\kappa\) chain (34). We constructed two vectors: the pCI/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/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...
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 ER were transiently cotransfected with pCI/GFP-ppins (lane 1) or pCI/ppins-GFP-ER (lane 2). At 36 h after transfection, cells were labeled for 12 h with 35S-methionine, immunoprecipitated with an anti-T mAb, and analyzed by SDS-PAGE and gel fluorography. The positions of Hsp73, T77-ppins, and cT272-A are indicated.

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-terminus (pCI/GFP-ppins) or the COOH-terminus (pCI/ppins-GFP-ER) of the ppins (Fig. 5A). In the later construct, the ppins-GFP sequence was further fused COOH-terminally with the ER retention signal (SEKDEL) to retard the recycling process that retrieves ER proteins back from the Golgi apparatus to the ER (30). The subcellular localization of the insulin/GFP fusion proteins was analyzed in HeLa cells transiently

FIGURE 5. Expression of GFP/ ppins fusion Ags. A, Schematic presentation 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, HEK cells transiently transfected with pCI/GFP-ppins or pCI/ppins-GFP-ER. Twenty-four hours after transfection, cells were fixed with 4% PFA, 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.
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 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 Kb/A12–21-specific IFN-γ+ 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 cytosolic/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 Kb/A12–21 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 Kb-restricted viral epitopes prevents priming of diabeticogenic CD8 T cells

The Kb/A12–21 epitope inefficiently stabilized Kb molecules on the surface of TAP-deficient RMA-S cells. This suggested that the Kb/A12–21 epitope has only low avidity for Kb (38) (Fig. 7A). To determine whether priming of Kb/A12–21-specific autoreactive CD8 T cell responses might be limited by “strong” Kb-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 targeting the Ag to the ER. Copriming CD8 T cells with Kb-restricted viral epitopes efficiently induced Kb molecules on the surface of TAP-deficient RMA-S cells. This suggested that the epitope competition for Kb molecules (32, 39, 40) may suppress ppins-specific T cell priming under these experimental conditions. This interpretation is supported by the observation that injection of a mixture of pCI/ppins and pCI/T77 vectors (encoding two well-defined D8- but no Kb-restricted epitopes (41, 42) or a mixture of pCI/ppins and pCI/GFP (encoding no H-2b-restricted epitopes) into the same muscle of RIP-B7.1 mice did not block EAD development (Fig. 7B, groups 2 and 3). These experiments confirmed that the failure to induce EAD by pCI/GFP-ppins and pCI/T77-ppins vectors depended on the cellular expression of the Ags rather than on immune-suppressive T cell epitopes in the respective Ags.
This study showed that ppins-encoding vectors efficiently induced K^b/A12-21-specific, diabetogenic CD8 T cells in RIP-B7.1 mice. In immunized RIP-B7.1 mice, professional APCs (processing ppins to pins in the ER after transfection with ppins-encoding DNA) have to present the A12-21 epitope to CD8 T cells. We investigated in vivo if ER-associated processing of ppins-derived Ags was essential to prime K^b/A12-21-specific CD8 T cells and leads to EAD. We found that small antigenic peptides (i.e., the A12-21 epitope and the A1-21 insulin A chain) that were targeted to the ER with the Ig signal sequence (pCI/L-A12-21, pCI/L-A1-21), but not the corresponding cytosolic peptides (pCI/A12-21, pCI/A1-21), could efficiently elicit K^b/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 K^b/A12-21 epitope were released within the ER (by signal peptidases) to load a critical number of the nascent K^b 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 proteosamal 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 K^b 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 K^b/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 K^b/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 K^b/ A12-21-specific CD8 T cells and triggered late EAD in RIP-B7.1 mice. This indicates that this epitope could be marginally presented 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, K^b/A12-21-independent EAD could be induced in RIP-B7.1 mice with a pCI/pins-A12-21 vector. This construct encoded an ER-associated pins without the K^b/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 K^b/A12-21 epitope could also be generated from different, COOH terminally extended polypeptides in the ER. We showed that the pins-GFP-ER fusion Ag (encoding the pins and thus the K^b/A12-21 epitope COOH terminally flanked by the GFP) or a pins-HA Ag (encoding the pins COOH terminally flanked by a nine-residue YPYDVPDYA hemagglutinin domain) (15) efficiently induced K^b/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 K^b/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 proteasomes (20, 21) but ER-resident peptidase subsets in these processes are yet not well understood (24, 25).

In nonpancreatic cells, ppins 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 (RK$_{55-56}$ and KR$_{48-49}$), 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-RK$_{mut}$ 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/ppins-RK$_{mut}$ 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$_{12-21}$ A$_{1-23}$) 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 re-stimulation of primed CD8 T cells requires high doses of the autologous 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 μg DNA) of ppins-encoding vectors. This confirmed that plasmid DNA immunization potently stimulates CD8 T cell responses in mice (48). DNA immunization induces Th1-biased immune responses to most Ags. In contrast, ballistic DNA immunization of low amounts (1–2 μg/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 β cell susceptibility to T cell-mediated death (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 β 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 β 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 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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