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Chaperones may cause the focus of diabetes autoimmunity on distinct (pro) insulin peptides

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ABSTRACT

It is still an enigma why T cell autoreactivity in type 1 diabetes targets few beta cell antigens only. Among these, one primary autoantigen is pro(insulin). Autoimmune T cells preferentially recognise three epitopes on the proinsulin molecule, of which the peptide region B:11-23 is the dominant one. Interestingly, the three regions superimpose with binding sites of the chaperone hsp70, the region B:11-23 being the strongest binding one. Absence of an intact core region B:15-17 prevents autoimmune diabetes in NOD as well as binding of hsp70. A role of hsp70 in selecting autoimmune epitopes is supported by the ability of this and other chaperones to deliver bound peptides to MHC class I and II molecules for efficient antigen presentation. Binding of hsp70 to receptors on antigen presenting cells such as TLR4 results in costimulatory signals for T cell activation. Strongest effects are seen for the mixture of hsp70 with the peptide B:11-23. Thus, hsp70 may assist in proinsulin epitope selection and efficient presentation to autoreactive T cells. The concept of chaperone guided immune reactivity may also apply to other autoimmune diseases.

1. Role of insulin as autoantigen in type 1 diabetes

One hallmark of type 1 diabetes is humoral and cellular immune reactivity to antigens expressed in beta cells of pancreatic islets. Among autoantigens, insulin is an important antigen to which autoantibodies can be demonstrated in the majority of children, preceding autoimmune reactivity to other beta cell antigens. Insulin autoantibodies prevail in children with the *DR4-DQ8* haplotype while autoantibodies to glutamic acid decarboxylase are more common than insulin autoantibodies in children with the *DR3-DQ2* haplotype and in adults [1–3]. Epitope mapping of autoimmune T cell reactivity to overlapping insulin peptides indicated that the most frequent epitope is in the region of residues B:10-24, in persons with type 1 diabetes [4]. The analysis of islet derived CD4 positive T cells from organ donors with type 1 diabetes of short duration demonstrated the presence of clones reactive with the B: 9-23 epitope [5]. Adoptive transfer of B: 9-23 specific T cells to humanised mice carrying the same HLA-DQ8 haplotype caused insulitis and diabetes [6]. Analysis of CD8 positive T cells autoreactive to proinsulin were mostly found *HLA-A2* restricted and preferentially recognised the same peptide region B:5-25 [7–9].

Insulin autoimmunity also plays a primary role in NOD mice which spontaneously develop autoimmune diabetes. Most islet infiltrating T cells were found to react to insulin, and the primary epitope recognised is the insulin B chain sequence 9-23. T cell clones with this specificity were able cause insulitis and diabetes after adoptive transfer to immunodeficient NOD *scid* mice [10]. NOD mice with a knockout of insulin 1 and 2 genes but carrying a proinsulin transgene in which B chain residue 16 within the region 9-23 was changed to alanine did not develop insulin autoantibodies, insulitis or diabetes [11].

2. Autoimmune diseases target selected autoantigens

It is still an enigma, why autoimmune diseases focus on a limited number of target antigens, which are often highly similar in men and mice with spontaneous autoimmune diseases, such as in autoimmune diseases of the thyroid, systemic lupus erythematosus, Sjögren's syndrome, myasthenia gravis, multiple sclerosis or autoimmune hepatitis [12–17]. As regards the pancreatic islet, why is it only the beta cell which is the target of destructive autoimmunity? Diseases with immune-mediated loss of alpha, delta, epsilon or gamma/PP cells are not

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known. Why, in beta cells, is insulin or proinsulin a dominant autoantigen? Finally, when looking at the (pro)insulin molecule, why is the peptide region B 9-23 of special importance for T cell autoreactivity, both in mice and men?

It is difficult to explain autoantigen selection at the level of class II MHC molecules only, because of their broad binding capacity for peptides. A recent analysis of the peptidomes of human MHC class II molecules expressed on cell lines identified more than 3000 peptides for HLA-DR and more than 7000 peptides for HLA-DQ [18]. There was no clear association between binding of beta cell epitopes to HLA-DQ8 and the ability of these peptides to elicit T cell responses [19]. Autoantigenic peptides from insulin often form only weak complexes with HLA-DQ8 or the corresponding MHC class II molecule $I–Ag^7$ of NOD mice [20,21]. This fits with a similar structure of peptide binding pockets of human DQ and mouse I-A proteins [22]. The peptidome of MHC class I from a beta cell line also was quite diverse, close to 3000 different peptides [8-12 mers] were eluted from purified HLA-peptide complexes of the haplotype *HLA-I* [9].

3. Possible role of chaperones in selecting autoantigenic peptides

In the case of insulin, we wish to discuss here the possible contribution of another peptide recognition system to the selection of amino acid stretches for presentation by MHC molecules to T cells. Antigen presenting cells (as other cells) express a second peptide recognition system, characterised by invariable receptors that have been well conserved during evolution. These receptors target a limited number of peptide sequence patterns of endogenous proteins. One common characteristic of these peptide sequences is that they must be chaperoned during protein synthesis to prevent improper folding or aggregation, or to reverse aggregation [23,24]. Binding properties of chaperones are well preserved from microbes to man, because of the necessity to rather selectively bind to those [hydrophobic] peptide stretches which are exposed during protein synthesis and might otherwise interact with the wrong peptide region [25,26]. This function is carried by several classes of chaperones that can be distinguished by their molecular weight and molecular structure [27]. Chaperones in the cytosol (hsp70 and 90) as well as in the ER (gp96 and calreticulin) have been found to bind peptides from antigenic proteins and to deliver such peptides to MHC class I or class II molecules, respectively [28]. In tumour immunology, heat shock proteins gp96, hsp 70, hsp90 and others have been isolated from tumour tissue and used as tumour peptide-chaperone complexes to elicit or enhance tumour-directed immunity [28–30]. Removal of bound peptides from heat shock proteins resulted in loss of immunogenic activity [29].

Heat shock proteins probably directly interact with MHC molecules. In vitro studies have demonstrated specific interaction of hsp70 with HLA-DR molecules, binding has been located to the ATPase domain of the heat shock protein which is outside the peptide binding groove involved in chaperoning. This suggests direct transfer of chaperoned peptides into the binding groove of HLA-DR [31]. Cross-presentation of hsp70 bound cytomegaloviral peptides to MHC class I molecules [HLA-A] has also been observed [32].

3.1. Hsp70 binds to selected regions of the insulin molecule

We analysed for a possible interaction of insulin with hsp70 by using in vitro binding assays of recombinant hsp70 of *E. coli* and (pre) proinsulin or a set of overlapping peptides. There were high affinity interactions with four regions of the preproinsulin molecule, the signal peptide and three discreet regions of the proinsulin molecule (B:9-25, C:15-31, A:6-21) [33]. Binding regions were characterised by a central hydrophobic leucine-rich core flanked by regions enriched for basic amino acids. These regions superimpose with the major target regions of MHC class II restricted T cell autoimmunity to proinsulin in type 1 diabetes and NOD mice [33]. Strongest binding was found for the insulin B chain peptide 11-23. Of particular importance appears to be the core region B:15-17. Changing the amino acid sequence B:15-17 from L-Y-L to L-A-L in transgenic NOD mice obliterated insulin autoimmunity and diabetes development [11]. Interestingly, the overlapping B chain peptide 18-30, which lacks the LYL motif, exhibited minimal binding to hsp70 [33]. The signal peptide and the B chain region 9-25 also superimpose with the dominant regions of CD8 restricted T cell autoimmunity [8,9].

The B:11-23 region is not readily accessible because it is located at the interface between two insulin heterodimers. This fits with the poor binding of intact insulin to bacterial hsp70. By contrast, there is good binding to non-oligomerised proinsulin [33]. Proinsulin is secreted from beta cells along with insulin. Hsp70 possibly may also be incorporated in secretory granules during proinsulin synthesis. Under conditions of metabolic or inflammatory stress larger amounts of proinsulin are released in relation to insulin [34–36]. In parallel, local concentrations of hsp70 are increased, within and outside cells, after secretion of the chaperone [37,38]. Hence, the interaction of hsp70 and proinsulin may occur within beta cells or in the extracellular environment. It is tempting to speculate that post-transcriptional modifications of the native insulin or proinsulin structure, as recently identified as target epitopes of autoimmune T cells [39–42], enhance binding to hsp70 or the subsequent transfer to the MHC peptide binding cleft.

The focus of the immune system on the B11-23 and two other regions of the proinsulin molecules may be aided by sequence similarities with microbes or viruses. A recent comprehensive research for the presence of viral peptide sequences with homology to 62 human peptide hormones identified cases of sequence similarities with 16 different hormones, including insulin [43]. There was no preferential homology of viral peptides with insulin. In four cases, homology with insulin and insulin-like growth factor was remarkable because the viral peptides (from members of the *Iridoviridae* family) contained all six cysteine residues critical for the insulin tertiary structure. However, none of these peptides had the B 15-17 sequence L-Y-L which has been reported essential for insulin diabetogenicity as described above [11] and for high affinity binding to hsp70 [33].

3.2. Binding of antigens to hsp70 promotes antigen uptake and presentation in APCs

Antigen presenting cells can take up exogenous hsp70 or hsp70-peptide complexes via several cell surface receptors including CD91, LOX-1 and the Siglec family [44–46]. In addition, there is a direct transfer of beta cell secretory vesicles or exosomes containing proinsulin to neighbouring macrophages or dendritic cells [47–49]. Exosomes are secreted by numerous cell types, and they have been found to contain hsp70 [50]. Beta cells may also secrete insulin fragments present in aged beta cell granules [crinosomes] [51] which may interact with extracellular hsp70. In all these possible pathways, endocytosis of hsp70-peptide complexes probably is followed by exposure to hydrolytic enzymes. However, the peptide sequence positioned in the hsp70 peptide binding cleft will not be easily accessible to proteolytic activity but is available for transfer to MHC class II molecules in the endosome compartment. I.e., after endocytosis of hsp70-peptide complexes there is effective antigen presentation on MHC class II molecules [52] (Fig. 1). In addition, hsp-bound antigenic peptides are also expressed on MHC class I molecules, by an intracellular process that is called cross-presentation [52].

3.3. Hsp70 delivers costimulatory signals to antigen presenting cells independent of peptide transfer

Effective antigen presentation of peptides delivered by exogenous hsp70 probably is further promoted by the property of hsp70 to mediate activation signals to macrophages or dendritic cells after binding to the surface of these cells. Exposure of antigen presenting cells to

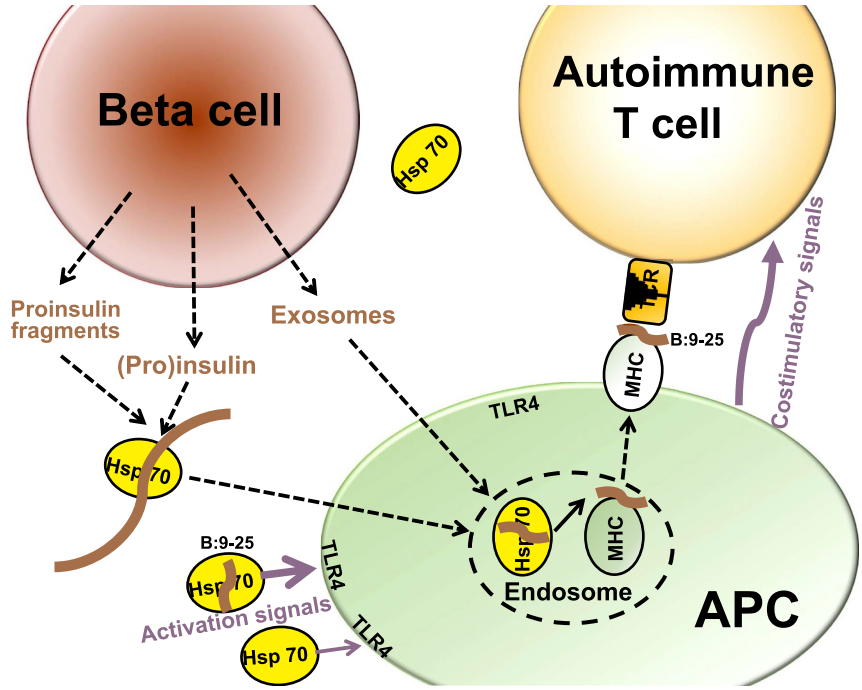

Fig. 1. Role of chaperone hsp70 in directing autoimmune reactivity. The chaperone hsp70 binds to the insulin B chain region B:9-25 when the latter is sterically accessible, as in proinsulin, in insulin fragments, in misfolded or modified insulins. Upon receptor-mediated uptake of hsp70-peptide complexes by antigen presenting cells (APC), the bound region B:9-25 is protected from hydrolytic attack and can be delivered to the MHC peptide binding cleft, followed by presentation on the APC surface, to T cell receptors (TCR) on autoimmune T cells. Extracellular complexes of hsp70 and insulin peptide region B:9-25 induce strong activation signals for APCs via the Toll-like receptor TLR4. As a consequence, APCs express costimulatory signal molecules in addition to presenting insulin peptides to autoreactive T cells.

extracellular hsp70 or hsp70-peptide complexes causes cell activation to release pro- and anti-inflammatory cytokines and there is upregulated expression of MHC class I and II molecules [30,53]. Activation of antigen presenting cells involves stimulation via TLR4 [54]. Because of this property, hsp70 and other chaperones are considered as danger signals, alarmins or damage-associated molecular patterns (DAMP). The ability to delivery costimulatory signals via engaging TLR4 may be of importance because this signalling pathway leads to potent activation of interferon-α gene expression [55]. As has been repeatedly observed, autoimmune reactivity is promoted by interferon-α [56], and a major role of this interferon in the pathogenesis of type 1 diabetes has been proposed [57].

Taken together, hsp70 may enhance steps critical for initiating an immune response to (pro)insulin, by “preselection” of autoantigenic peptide sequences followed by transfer to MHC molecules in antigen presenting cells, and the delivery of costimulatory signals. The sequence pattern of amino acids recognised by hsp70 is present also on other proteins present in beta cells. However, proinsulin is the major peptide synthesised when cells are exposed to insulin secretagogues or low levels of inflammatory mediators [35,58,59]. Viral infections cause insulin resistance [60] and thus will also increase the amount of proinsulin released. Beta cell stress appears to precede and accompany immune-mediated beta cell destruction in pre type 1 or autoimmune diabetes [36,61–63], and cell or organ stress usually is associated with enhanced local production and release of hsp70 and other stress proteins [53,64,65]. Under such conditions, enhanced local concentrations of hsp70 probably promote a pro-inflammatory milieu as well as autoantigen presentation.

3.4. Insulin peptide B:11-23 enhances the costimulatory potency of hsp70

As described above, binding of proinsulin to hsp70 (from *E. coli*) occurs at three regions, at peptides B:9-25, C:15-31 and A:6-21 which are identical to the major targets of T cell autoimmunity in type 1 or autoimmune diabetes [33]. Is there a difference in the interaction of peptides from the three regions with hsp70 which might help to understand the dominance of the B:9-25 region as autoantigen? Measurements of binding affinity indicated that peptides from the B:9-25 region exhibited stronger binding to hsp70 than peptides from the C and A region. However, one peptide from the A region [peptide A:8-20] almost matched the binding strength of the strongest binding peptide from the B region (peptide B:11-23) [33]. Another possibility is that the binding of peptides to hsp70 impacts the costimulatory activity of the stress protein. Indeed, when hsp70 was added to mouse or human macrophages at a concentration below the threshold for eliciting a pro-inflammatory cytokine response, admixture of peptide B:11-23 caused macrophage activation, as documented by the release of TNFα, IL-β and IL-6. Incubation of macrophages with hsp70 or peptide B:11-23 alone did not cause cytokine release [66]. No significant cytokine secretion was observed when macrophages were incubated with mixtures of hsp70 and peptides from the C or A regions. I.e., binding of peptide B:11-23 appears to enhance the costimulatory potency of hsp70, by an unknown mechanism.

These findings suggest that “preselection” of the autoantigenic insulin peptide region B:9-25 by hsp70 for presentation by MHC molecules may include both, efficient binding to the peptide binding site of the chaperone and enhanced costimulatory activity of the hsp70-peptide complex (Fig. 1). We assume that the two properties contribute to the autoantigenic dominance of the peptide region B:9-25 in type 1 diabetes.

4. Role of chaperones in autoimmune reactivity to other islet autoantigens

Little is known about the possible role of chaperones in selecting T cell epitopes of other relevant beta cell autoantigens in type 1 diabetes, glutamic acid decarboxylase (GAD), insulinoma associated antigen 2A (IA-2A) or the zinc transporter T8 (ZnT8). Several peptide regions of GAD serve as HLA-restricted epitopes for T cells. The most important region is GAD113-132 to which both CD4 and CD8 positive T cells show autoreactivity in type 1 diabetes [67,68]. Interestingly, this peptide harbours a leucine rich core with some flanking basic amino acids, as is characteristic for the binding motif of DnaK and hsp70 [69]. However, binding studies of this peptide to DnaK or hsp70 have not been conducted.

Whether autoimmunity-relevant epitopes of IA-2A or ZnT8 correspond to binding motifs of chaperones remains to be analysed. There are additional chaperones in addition to hsp70 which have been found

to bind antigenic peptides and guide/promote immune reactivity, such as hsp60, hsp90 and several endoplasmic reticulum chaperones such as gp96 or BiP/Grp78 [28,30,70]. Several chaperones such as hsp60, hsp90 or gp96 share with hsp70 the property of delivering costimulatory signals to antigen presenting cells [30,64,71,72]. Chaperones therefore may promote autoreactivity to other islet antigens than insulin, in conjunction with appropriate HLA haplotypes conferring susceptibility for type 1 diabetes.

4.1. Role of chaperones in assisting autoantigen selection in other autoimmune diseases

If the transfer of endogenous peptides from chaperones to MHC molecules is of relevance for guiding autoimmune reactivity, this should be the case in other immune-mediated diseases. This hypothesis has not been well researched. In a study of experimental autoimmune encephalomyelitis, antigen presenting cells were pulsed with myelin basic protein which resulted in complexes of the autoantigen with hsp70. Hsp70 selectively bound to three peptide regions of myelin basic protein and these included the two immunodominant epitopes MBP 85-99 and 80-99. Complexes of hsp70 with myelin basic protein enhanced responses of a T cell hybridoma specific for MBP80-99 [73]. In rheumatoid arthritis, chaperone Grp78 was found to bind an autoimmune epitope of Ro [SS-A], Ro52 378–391 [74]. The chaperone calreticulin rather selectively bound to the two epitopes of the Ro60 kDa autoantigen, spanning the sequences 175–184 and 216–232 [75]. Whether these interactions promote autoimmune reactivity remains to be analysed.

5. Concluding comments

It is still an enigma why, in autoimmune diseases, T cells only target a limited number of autoantigens and a limited number of autoantigen epitopes, despite the broad binding repertoire of MHC molecules and of T cell receptors. The data presented here allow the suggestion that chaperones contribute to this phenomenon. Chaperones have phylogenetically well-preserved binding sites for peptide regions important for proper protein folding and function. Many chaperones can deliver bound peptides to MHC class I and class II molecules, together with costimulatory signals. This may guide and enhance autoantigen presentation under conditions of increased chaperone function, i.e. during cell or organ stress.

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Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.102304.

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