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Granzyme B cleavage of autoantigens in autoimmunity

Erika Darrah[1] and **Antony Rosen**[1,*]

[1]Division of Rheumatology, Department of Medicine, Johns Hopkins University School of Medicine

Abstract

The systemic autoimmune diseases are a complex group of disorders characterized by elaboration of high titer autoantibodies and immune-mediated damage of tissues. Two striking features of autoimmune rheumatic diseases are their self-sustaining nature and capacity for auto-amplification, exemplified by disease flares. These features suggest the presence of a feed-forward cycle in disease propagation, in which immune effector pathways drive the generation/release of autoantigens, which in turn fuel the immune response. There is a growing awareness that structural modification during cytotoxic granule-induced cell death is a frequent and striking feature of autoantigens, and may be an important principle driving disease. This review focuses on granzyme B (GrB)-mediated cleavage of autoantigens including (*i*) features of GrB cleavage sites within autoantigens, (*ii*) co-location of cleavage sites with autoimmune epitopes, and (*iii*) GrB-sensitivity of autoantigens in disease-relevant target tissue. The mechanisms whereby GrB-induced changes in autoantigen structure may contribute to the initiation and propagation of autoimmunity are reviewed and reveal that GrB has the potential to create or destroy autoimmune epitopes. As there remains no direct evidence demonstrating a causal role for GrB-cleavage of antigens in the generation of autoimmunity, this review highlights important outstanding questions about the role of GrB in autoantigen selection.

Keywords

granzyme; autoimmunity; autoantibody; autoantigen; antigen processing; proteolysis

Features of Systemic Autoimmune Disease

The systemic autoimmune diseases are a complex group of disorders which include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), autoimmune myopathies, Sjögren's syndrome (SS), vasculitis and scleroderma. The diseases manifest in a wide range of clinical phenotypes, often united by chronic inflammation and involvement of multiple organ systems. A dominant feature of these diseases is the elaboration of high titer, high affinity autoantibodies, which are strongly associated with phenotype (1-3). While the clinical presentations of the individual systemic autoimmune diseases are often quite distinct, the presence of overlapping features in different diseases suggest the involvement of common effector pathways and shared disease mechanisms (4). This includes autoimmune myopathies occurring in myositis, SLE and scleroderma, or the *sicca* complex (dry eyes/mouth) in many of the systemic autoimmune diseases. Autoantigens in systemic autoimmune diseases appear to be ubiquitously-expressed molecules and may be central to

Corresponding author: Antony Rosen, MB ChB, The Johns Hopkins School of Medicine, arosen@jhmi.edu; Erika Darrah, BS, The Johns Hopkins School of Medicine, edarrah1@jhmi.edu; Potential referee: Nancy A. Thornberry, Department of Metabolic Disorders, Merck Research Laboratories, Rahway, NJ 07065, Nancy_Thornberry@merk.com. *Corresponding author: Antony Rosen, MB ChB, Division of Rheumatology, Johns Hopkins University School of Medicine, Mason F. Lord Building, Center Tower, Room 412, Baltimore, MD 21224. Phone: 410-550-1900; Fax: 410-550-2072; arosen@jhmi.edu.

the disease process, but the mechanisms underlying their selection remain unclear. Defining the features unifying these autoantigens, which may impact their selection by the immune response, is an important priority.

One striking feature of autoimmune rheumatic diseases is their self-sustaining nature, and their striking capacity for auto-amplification (exemplified by disease flares). The autoantibody response in active disease is typically of high affinity and titer, and appears to be driven by ongoing antigen release. This amplification strongly suggests the presence of a feed-forward cycle in disease propagation consisting of two key components: active immune effector pathways and immunogenic self antigens. Immune effector pathways drive the generation/release of autoantigens from target tissue, which in turn provide fuel to propagate the immune response. Autoantigens are not passive players in this process but rather have a direct role in shaping the autoimmune response. Identifying such amplifying pathways may have important therapeutic implications.

There is a growing awareness that structural changes of autoantigens during cytotoxic lymphocyte granule-mediated cell death are a frequent feature of systemic autoimmune diseases. Although this review focuses on structural changes of intracellular autoantigens induced by the granule component granzyme B (GrB), it is important to note that there are several other mechanisms by which cytotoxic granules may contribute to the self-sustaining cycle of tissue damage in autoimmune disorders. First, structural modification of an overlapping yet distinct set of autoantigens has been demonstrated for other members of the granzyme family including granzyme A (GrA) (5, 6) and granzyme H (GrH) (7). Second, granzymes can mediate direct extracellular effects on target tissue. Neurotoxicity is induced following incubation of neurons with GrB via G-coupled protein receptor signaling and may have a role in the pathogenesis of neuroinflammatory diseases (8). Additionally, degradation of extracellular matrix proteins by GrB is thought to play a role in cytotoxicity of smooth muscle cells in inflammatory vascular disease (9, 10). Finally, a role for granzymes as independent pro-inflammatory modulators of antigen-presenting cell (APC) function has been suggested by the finding that mouse macrophages treated with GrA secrete pro-inflammatory cytokines IL-1β, TNFα, and IL-6 (11). This review discusses GrB-mediated changes in autoantigen structure and mechanisms whereby these changes may contribute to the initiation and propagation of autoimmunity.

Granzymes modify the structure of autoantigens during cytolytic granule-mediated cell death

During lymphocyte-induced cytotoxicity, granzymes and the pore-forming protein perforin act in concert to provide protease access to the target cell cytosol. Granzymes induce cell death by several processes, including activation of the caspase cascade, as well as direct cleavage of various intracellular substrates that participate in nuclear fragmentation and cell death (12). Significant data exists to suggest an important role for the cytotoxic lymphocyte granule pathway in the pathogenesis of systemic autoimmunity. Activated cytotoxic lymphocytes are enriched in the lung in scleroderma (13, 14), the salivary gland in SS (15), and the synovium in RA (16). In the muscle of individuals with polymyositis (PM), T cells in direct contact with muscle fibers have been shown to orient components of the cytolytic granule pathway toward the site of cell-cell contact (17). Cytolytic CD8+ T cells invading muscle fibers of PM patients exhibit a restricted T cell receptor repertoire suggesting clonal expansion of autoantigenic T cells in the inflamed target tissue (18). Furthermore, increased circulating levels of cytolytic cells and components of the granule pathway correlate with disease activity. For example, the number of circulating activated cytolytic CD8+ T cells correlates with disease activity in SLE (19), while the levels of GrB in the serum and

synovial fluid of RA patients is strikingly associated with the severity of erosive joint disease (20, 21).

Multiple studies have demonstrated that the majority of autoantigens targeted in systemic autoimmune diseases are substrates for granzymes, particularly GrB (5, 6, 22-24) (Table 1). The association between GrB cleavability and autoantigen status is striking since, until recently, there have been few GrB substrates identified which are not autoantigens. A recent comprehensive proteomic analysis by Van Damme *et al* revealed hundreds of novel GrB targets, and may suggest a pool of previously undefined autoantigens (25).

The tetrapeptide substrate specificity of human GrB has been determined. In addition to the requirement for aspartic acid in the P1 position, P4 is specific for isoleucine (Ile), valine (Val) and leucine (Leu), while P3 and P2 are able to accommodate a wider range of amino acids (26, 27) (Figure 1A). While there are many tetrapeptides satisfying the GrB consensus sequence within molecules, most of these sites are not cleaved by GrB (28). Indeed, GrB typically cleaves autoantigen substrates at a limited number of sites (22). Interestingly, although GrB has similar tetrapeptide substrate specificity to the upstream group III caspases (which process effector caspases 3 and 7) (27), distinct sites are utilized generating unique fragments of autoantigens not seen during caspase-mediated cell death (22).

The GrB cleavage sites defined within autoantigens reveal several interesting features (Figure 1B). First, all have Ile, Leu, or Val in P4, and are cleaved after the P1 aspartic acid (22). Second, amino acids in the P2 and P3 positions in autoantigens are almost universally favored by human GrB but not tolerated by caspases (e.g. proline in P2) (26). Finally, the native three-dimensional antigen structure may determine GrB cleavage site accessibility. For example, while GrB is able to cleave an *in vitro* generated autoantigen (Sjögren's syndrome transmembrane autoantigen, muscarinic receptor 3) at two distinct sites, only one site is utilized during granule-induced killing of intact human salivary gland target cells (23) (25). These features reinforce the strong relationship between autoantigen status and this unique cleavage susceptibility.

Not unexpectedly, granzyme cleavage of antigens frequently has consequences in terms of function (5,24), including redistribution of nuclear antigens to the cytosol during cytotoxic granule-induced cytotoxicity (6,29). The SS nuclear autoantigen La/SSB is a substrate for both GrB and GrH, and undergoes dramatic redistribution during granule-mediated cell death following uncoupling the C-terminal nuclear localization signal (NLS) (29). Similarly, cleavage of SLE and autoimmune hepatitis antigen lamin B by GrA and B causes disruption of the nuclear lamina, by uncoupling lamin B from its NLS (6). The granule pathway has also been shown to trigger the release of autoantigens from target cells. Blanco *et al* demonstrated that cytolytic CD8+ T cells from patients with active but not quiescent SLE were able to stimulate the release of nucleosomes from target cells, together with cleavage of nuclear autoantigens (e.g. U1-70kDa) (19). It is conceivable that cytoplasmically redistributed granzyme-cleaved antigens are released during granule-induced death, potentially influencing pathways of antigen presentation.

The ability of GrB to modify the structure of self antigens is not limited to systemic autoimmunity. There are examples of tissue-specific (28,30) and cancer autoantigens being GrB substrates (31). Myasthenia gravis (MG) is a tissue-specific autoimmune disease mediated by the antibody-dependent reduction of acetylcholine receptors (AchR) at the neuromuscular junction (32). Both GrB and its substrate (AchR ε subunit) are aberrantly expressed in thymuses from patients with MG, suggesting that an immune response to AchR may propagate in the thymus (30). In medullary carcinoma of the breast (MCB), GrB expressing cytotoxic T cells are found in large numbers, often nearby apoptotic tumor cells.

Autoantibodies to B-actin have been identified in these patients and are able to recognize actin that has been redistributed to the surface of apoptotic tumor cells. GrB cleavage fragments of β-actin have been detected in MCB tumor lysates, suggesting that CTL-mediated death of MCB tumor cells and actin redistribution may play a role in the generation of autoimmunity to β-actin (31). Interestingly, patients with MCB have a significantly higher survival rate than patients with non-MCB breast cancer, suggesting that autoimmunity generated against tumor self-antigens may contribute to the favorable prognosis (33, 34).

These data demonstrate that a group of molecules which share little in common in terms of structure, function, or distribution, are unified by their susceptibility to cleavage by a highly specific and fastidious protease that has a major role in immune effector pathways. In order to place in context the possible relevance of GrB-mediated cleavage of autoantigens to initiation and propagation of autoimmunity, it is necessary to review the concepts of immunodominance and the ability of single, early proteolytic events to influence epitope selection.

Protein structure and early proteolytic events influence epitope selection during antigen presentation

Structural modification of autoantigens including protein-protein interactions, post-translational modifications, mutation of key residues, and differential sensitivity to proteases can lead to striking changes in the peptides that are ultimately presented to T cells (35). Molecules that are found in, or gain access to the extracellular space (including those derived from dying cells), are phagocytosed by professional antigen presenting cells (APCs) and gain access to the MHC class II antigen processing pathway. Upon entry, antigens are exposed to a variety of proteases in the endosomal compartment, which process the native antigen into peptide fragments, available for loading onto class II molecules. Interestingly, although a large number of potential peptides can be generated, only a few peptides are successfully presented on the surface of the APC. These peptides are termed ‘immunodominant’ (36).

There is accumulating evidence to suggest a role for protein conformation and MHC-guided processing as one mechanism underlying immunodominance (37). During antigen processing, exposed structural determinants of native antigens can bind to MHC class II molecules and be protected from proteolysis by endosomal cathepsins, while the rest of the molecule is degraded. Alterations in antigen structure could therefore have profound effects on the regions of the protein available for binding to, and protection by, class II molecules. During development of immune tolerance, T cells with high affinity for dominant peptides derived from self-proteins are often deleted or rendered non-responsive (38). Those peptides that are not presented on class II molecules at significant levels are termed ‘cryptic’ and T cells recognizing such cryptic determinants are not tolerized and persist. Differences in antigen processing of self proteins occurring as a result of structural modification could enhance cryptic epitope presentation, leading to activation of self-reactive T cells (36). Activation of T cells recognizing cryptic epitopes has been proposed to be an important mechanism underlying the development of autoimmunity (39).

Recent studies demonstrating the importance of early and highly specific proteolytic events in presentation of immunodominant and cryptic epitopes are highly relevant to the observation that autoantigens are proteolyzed in a novel way by granzymes. For example, asparaginyl endopeptidase (AEP) is a cysteine protease involved early in MHC class II antigen processing. Cleavage of intact endocytosed protein by AEP occurs at a limited number of asparagine residues, yet has profound effects on downstream processing by

lysosomal proteases (40). Thus, cleavage of tetanus toxoid C fragment (TTCF) at a single asparagine residue by AEP is critical for presentation of multiple peptide epitopes derived from other regions of the protein. Mutation of the TTCF AEP cleavage site alters the pattern of fragments generated during subsequent processing and dramatically reduces activation of several antigen-specific T cell hybridomas (41).

Processing by AEP has been implicated in the generation of autoimmunity. For example, a major disease-associated epitope in the multiple sclerosis autoantigen myelin basic protein (MBP) contained within amino acids 84-102 is normally cryptic, due to proteolysis at Asn94 by AEP. Inhibition of AEP activity in APCs enhances presentation of this epitope, which is a dominant epitope when autoimmunity to MBP is subsequently initiated (42). Additionally, proteolytic activity of AEP is impaired when asparagine residues are N-glycosylated, suggesting a way in which post-translational modifications can affect susceptibility to proteolysis and subsequent antigen presentation (40).

In this regard, it is notable that antigen proteolysis and epitope selection is also influenced by subtle changes in antigen structure and conformation in several models of autoimmunity. For example, the post-translational conversion of aspartic acid to isoaspartyl residues is an isomerization that occurs naturally during cell stress, aging, and activation. The presence of isoaspartyl residues in full-length pigeon cytochrome C alters processing by cathepsin D and stimulates T cell proliferation. However, the aspartyl is not stimulatory, suggesting that accumulation of a naturally occurring amino acid modification can render self antigens immunogenic (43). Immune responses to isoaspartyl, but not aspartyl, forms of the SLE autoantigen U1/Sm ribonucleoprotein have also been observed. Interestingly, activated B and T cells contain increased levels of isoaspartyl residues and may act as sources of autoantigen to drive the self-sustaining loop of autoimmunity (44). Furthermore, modification of single amino acid residues within endogenous proteins can generate autoimmunity to cryptic epitopes. For example, dopachrome tautomerase (Dct) is an antigen normally expressed in melanocytes. When randomly mutated constructs of Dct are expressed in mice, autoimmunity to Dct is induced, resulting in skin hypopigmentation and activation of autoreactive T cells recognizing cryptic Dct epitopes. Autoreactive T cells and hypopigmentation are also observed when the Dct constructs encoding truncated versions of the molecule, not generated during normal processing of the full length protein, are expressed (45).

Taken together, these studies demonstrate that relatively minor changes in autoantigen structure, including single proteolytic events, can have striking effects on epitope selection during antigen processing and presentation on MHC class II molecules. Autoimmunity may arise when normally dominant epitopes of self antigens are destroyed or cryptic epitopes are revealed.

Conformational state of antigens affects susceptibility to granzyme B cleavage

As noted above, proteolysis and epitope selection can be affected by minor changes in antigen structure, including post-translational modifications (40), isomerizations (43), and early cleavage events (41). Several studies have demonstrated that the tissue-specific expression (46-48) or conformation (49, 50) of proteins can affect their susceptibility to cleavage by GrB. For example, GrB cleavage of extracellular matrix proteins (vitronectin and fibronectin) and the clotting mediator, von Willebrand factor, only occurs when the proteins are in distinct matrix-associated conformations (49, 50). These studies suggest that autoantigens may be susceptible to GrB cleavage only under very specific circumstances associated with unique activation, differentiation, or mitotic states of the cell (Figure 2).

Granzyme-sensitive forms of autoantigens have been observed in the disease-relevant target tissue in myositis and scleroderma. For example, expression of a GrB-sensitive form of histidyl-transfer RNA synthetase (HRS/Jo-1), a target antigen in patients with interstitial lung disease associated with myositis (51), has also been noted exclusively in lung tissue as compared to other tissues including placenta, liver, muscle, ovary and colon. The exclusive expression of granzyme-sensitive HRS/Jo-1 in the lung (which is the target of immune-mediated damage in this disease) is of interest. It is consistent with the proposal that GrB-mediated cleavage plays a role in antigen selection, and that expression of cleavable autoantigens in specific target tissues may focus amplifying injury at that site (46). A similar tissue-specific expression of a granzyme-sensitive form of the nucleolar autoantigen nucleophosmin (B23) has been observed in scleroderma. Autoantibodies to B23 are found in the serum of scleroderma patients, where they are associated with the development of pulmonary hypertension in which vascular smooth muscle cell damage is believed to play a role in pathogenesis (52). Interestingly, B23 from differentiated but not undifferentiated vascular smooth muscle cells is efficiently cleaved by GrB. The restricted expression of cleavable autoantigens to particular cells in specific differentiation states within the target tissue suggests that either the property of cleavability or cleavage events themselves may be relevant to immune-mediated damage (47).

B23 is also a target antigen in patients with hepatocellular carcinoma (HCC) (53). Unlike normal regions of liver tissue, tumorous areas of liver also express a highly cleavable form of B23, which exists primarily as an SDS-stable oligomer. This cleavable form of B23 is preferentially recognized in HCC tumors by an antibody specifically raised to a peptide within the GrB cleavage site, suggesting that HCC liver expresses a form of B23 in which the GrB cleavage site is exposed, rendering it more sensitive to cleavage (48).

Distinct or aberrant post-translational processing of autoantigens may also affect susceptibility to cleavage by GrB (Figure 2). The glutamate receptor subunit 3 (GluR3) is an autoantigen in the tissue-specific autoimmune disease Rasmussen’s encephalitis (54). The glycosylation state of GluR3 appears to be critical in determining susceptibility to GrB cleavage. While the N-glycosylated granzyme-insensitive form predominates in neuronal tissue, a percentage is not glycosylated and is therefore susceptible to cleavage by GrB. It has been proposed that the balance of the glycosylated versus unglycosylated forms is modified during inflammation, potentially providing an opportunity for GrB-mediated cleavage only under very limited circumstances (28).

Together, these data highlight that changes in protein conformation affect susceptibility to GrB cleavage in target tissues in autoimmunity as well as cancer. The association between status as an autoantigen and susceptibility to cleavage by GrB is striking, as are the clear examples where the cleavable conformation of an autoantigen is enriched or expressed exclusively within the target tissue associated with an immune response to that antigen. Taken together, the data implicate novel cleavage events directed by immune effector pathways, occurring at specific tissue sites, as components of the pathways selecting self antigens in autoimmune rheumatic diseases. It must be noted however, that there is to date no direct evidence that GrB cleavage of autoantigens alters the immune response to that antigen, or participates in disease propagation. It is possible that cleavable forms of antigens exist in disease relevant target tissues and represent an altered, immunogenic conformation that can drive the generation of autoimmunity even in the absence of GrB cleavage.

Unfortunately, both the tetrapeptide and the macromolecular specificities of human GrB differ significantly from mouse GrB, and the unique unifying features around GrB cleavage sites in human autoantigens are largely unshared by mouse antigens (55-57). Defining the role of GrB in human systemic autoimmune diseases therefore depends on addressing the

effects of GrB cleavage events on antigen immunogenicity in the human model – which constitutes a significant challenge. Possible ways around this barrier include: (*i*) examining effects of cleavage with human GrB (or fragments resulting from such cleavage) on immunogenicity of defined human autoantigens in mice; (*ii*) studying initiation and propagation of various forms of systemic autoimmunity in a GrB knock-out mouse or in a human GrB knock-in mouse; and (*iii*) directly testing effects of GrB on autoantigen immunogenicity and epitope selection in peripheral blood from humans with autoimmune diseases in which GrB substrates are targeted.

Limited studies have been performed in this regard and suggest conflicting roles for GrB cleavage of antigens in the generation of autoimmunity. Mouse experiments have been performed which suggest that fragments of antigens elicit more robust immune responses than intact protein (45, 58). For example, T cells from transgenic mice expressing human MHC class II molecules (DRB1*0301/DQB1*0201) associated with the development of immune responses to SLE autoantigen La, exhibit enhanced T cell proliferation upon peptide re-challenge after priming with a truncated from of the protein. This naturally occurring frame-shift mutant (amino acids 1-204) is very similar in length to the N-terminal fragment of La generated after GrB cleavage (amino acids 1-220) suggesting that fragmentation of La may enhance antigen processing and release of cryptic epitopes (58). A study in GrB knock-out mice shows that GrB is not required for the development of pristane-induced SLE and may even have a protective effect. GrB knock-out mice injected with pristane exhibited features of SLE, including autoantibody production, but appeared to have increased mortality after pristane treatment, compared to wild-type mice (59). Experiments in human GrB knock-in mice may provide insights into the effect of autoantigen cleavage in a mouse model of autoimmunity, provided that the GrB cleavage site within the autoantigen is conserved between mice and humans. It is clear that future studies are necessary to elucidate the potential role for GrB cleavage of autoantigens in the generation of autoimmunity and will require studies in the human model.

Granzyme B cleavage sites reside in unstructured regions of antigens and co-locate with autoimmune epitopes

The ability to obtain detailed information about the structure of autoantigens by crystallography as well as by dynamic methods including protein nuclear magnetic resonance (NMR) and deuterium exchange mass spectrometry (DXMS) may provide valuable information regarding the three-dimensional context of granzyme cleavage sites. Although, this data is available for very few antigens at this time, some interesting themes are emerging. Analysis of the available crystal structures of autoantigens known to be cleaved by GrB reveals a common features of granzyme cleavage sites (60, 61). Partial structures of B23 (62), poly(ADP)ribose polymerase 1 (PARP1) (63), and HRS/Jo-1 (64) demonstrate that GrB cleavage sites are located in unstructured regions or in areas adjacent to highly structured elements such as coiled-coils. Similarly, the GrB site ($IEAD^{15}$) in the scleroderma antigen and DNA replication protein topoisomerase I is located in the highly extended 174 amino acid N-terminal domain. This region was determined by circular dichroism to be virtually completely unfolded (65). In other cases, known granzyme cleavage sites are located on loosely ordered surface loops or linker regions between structural elements. The structure of scleroderma autoantigen fibrillarin reveals that the GrB cleavage site ($VGPD^{184}$) is located on a surface loop between an α-helix and β-strand (66).

The effect of antigen structure on epitope selection is highlighted by work from Landry *et al* which showed that disordered regions of antigens lead to increased presentation of epitopes from adjacent, structurally organized sequences (67). Since GrB cleavage sites tend to be located in disordered areas or linker regions connecting structural elements, it is possible

that cleavage of autoantigens at the GrB site influences the presentation of downstream or hidden structured epitopes (Figure 3). Again, a complete dataset is not available, but review of cleavage sites as well as known B and T cell epitopes of autoantigens reveals that granzymes and the immune system target overlapping regions of these proteins (Figure 4). Epitope mapping of La has revealed overlapping B (68) and T cell (58, 69) epitopes in a region spanning the GrB cleavage site. Similarly, overlapping B (70) and T cell (71) epitopes have been identified in the SLE autoantigen U1-70kDa in a C-terminal region containing the GrB cleavage site.

While the discussion of the effect of autoantigen cleavage by granzymes has focused on antigen processing and presentation of peptide epitopes to T cells, it is clear that autoantibody epitopes can also be affected. Granzyme cleavage of autoantigens can lead to the creation of immunogenic protein fragments which are preferentially recognized by autoantibodies from patients with systemic autoimmune diseases (72, 73). Autoantibodies from some patients with primary Sjögren's syndrome have been shown to preferentially recognize GrB cleavage fragments of La/SSB. A subset of these patients has autoantibodies that recognize cryptic epitopes generated following GrB cleavage, in that they cannot be blocked by intact La protein (72). Likewise, sera from patients with scleroderma and ischemic digital loss exhibit a striking preference for GrB-generated fragments of antigens, specifically those with autoantibodies to centromere protein CENP-C (73).

Although cleavage of autoantigens by granzymes can aid in the generation of antigenic epitopes, granzyme cleavage also has the capacity to destroy immunoreactive epitopes. Circulating autoantibodies (74) and T cells (75) targeting pyruvate dehydrogenase complex-E2 (PDC-E2) are a frequent finding in patients with primary biliary cirrhosis (PBC). While cleavage of PDC-E2 by caspases is capable of generating immunogenic fragments, GrB cleavage appears to destroy a dominant autoantibody epitope, abolishing reactivity by PBC patient sera (76). Interestingly, while two CD8+ T cell epitopes may be destroyed by GrB cleavage (77, 78), another (79) appears to be unaffected and perhaps even liberated by proteolysis. A similar pattern is observed for MG autoantigen AchRε, in which GrB cleavage has the potential to destroy a T cell epitope while liberating another (80).

Conclusions

There is strong evidence implicating the cytotoxic lymphocyte granule pathway in the pathogenesis of systemic autoimmune diseases. Activated cytotoxic lymphocytes are present in target tissues and effector function is positively correlated with disease activity in several diseases (e.g. myositis, SLE, RA). Autoantigens have diverse structures, functions, and subcellular localizations yet are efficiently cleaved by GrB at a small number of unique sites, distinct from caspase cleavage sites. GrB-sensitive conformations of antigens have been detected in the disease-relevant target in systemic autoimmune diseases. Analysis of available data demonstrates that GrB cleavage sites are often situated in unstructured regions of antigens, adjacent to structured domains. Immune epitopes are also frequently located in close proximity to GrB cleavage sites, suggesting that cleavage by GrB may modulate presentation of epitopes from adjacent structured regions. Although there is clear data that single proteolytic events early during antigen processing can have striking effects on the epitopes presented, such data is not yet directly available for GrB. The observation that human and mouse GrB have distinct cleavage site specificities underscores the challenges to demonstrate a direct role for GrB-mediated cleavage of autoantigens in human autoimmunity. Studies in the human system will be needed, and will require an antigen which is frequently targeted in a phenotypically distinct autoimmune disease, a substrate for human GrB, and associated with known MHC class II alleles. Establishing a mechanistic

role for granzymes in the self-amplifying pathway of autoimmunity would have important therapeutic implications.

Abbrevations

SLE	systemic lupus erythematosus
RA	rheumatoid arthritis
SS	Sjögren's syndrome
GrB	granzyme B
GrA	granzyme A
GrH	granzyme H
APC	antigen-presenting cell
PM	polymyositis
Ile	isoleucine
Val	valine
Leu	leucine
NLS	nuclear localization signal
MG	myasthenia gravis
AchR	acetylcholine receptor
MCB	medullary carcinoma of the breast
MHC	major histocompatibility complex
AEP	asparaginyl endopeptidase
TTCF	tetanus toxoid C fragment
MBP	myelin basic protein
Dct	dopachrome tautomerase
HRS/Jo-1	histidyl-transfer RNA synthetase
HCC	hepatocellular carcinoma
GluR3	glutamate receptor subunit 3
NMR	nuclear magnetic resonance
DXMS	deuterium exchange mass spectrometry
PDC-E2	pyruvate dehydrogenase complex-E2
PBC	primary biliary cirrhosis

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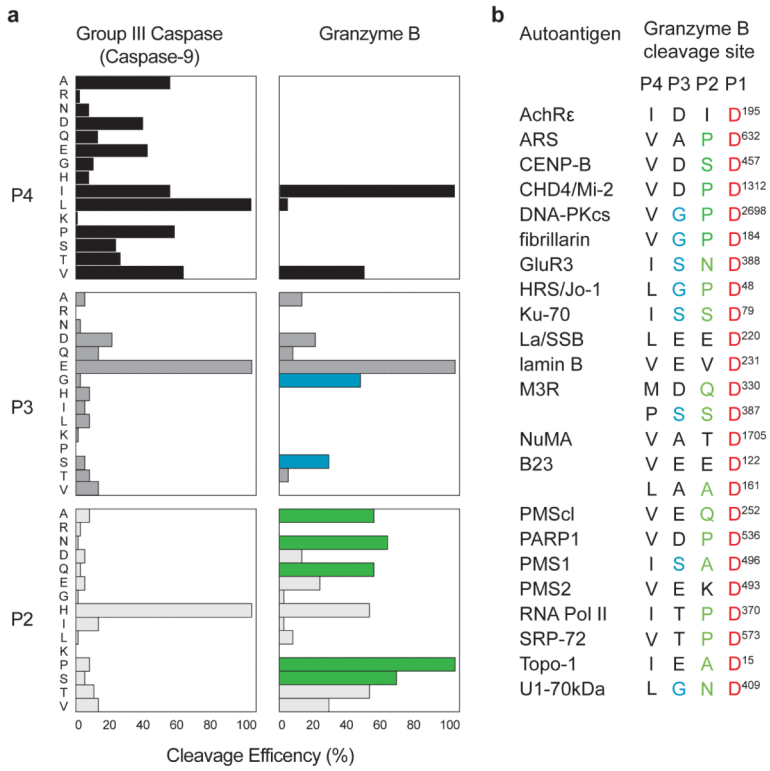

Figure 1. Cleavage sites in autoantigens are enriched in amino acids specifically preferred by GrB over group III caspases

(A) Specificities of GrB and group III caspases are similar but distinct. A comparison of the P2, P3, and P4 position requirements for a group III caspase (caspase-9) and GrB reveals differences in preferred amino acids (modified from Thornberry *et al* (22)). (B) Cleavage sites of autoantigens known to be GrB substrates are enriched in amino acids that are preferred by GrB in the P2 and P3 positions over caspase-9. Amino acids preferred by GrB are shown in green for the P2 and in blue for the P3 positions. Amino acids are indicated as follows: A,alanine; R,arginine; N, asparagine; D, aspartic acid; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; K, lysine; P, proline; S, serine; T, threonine; V, valine.

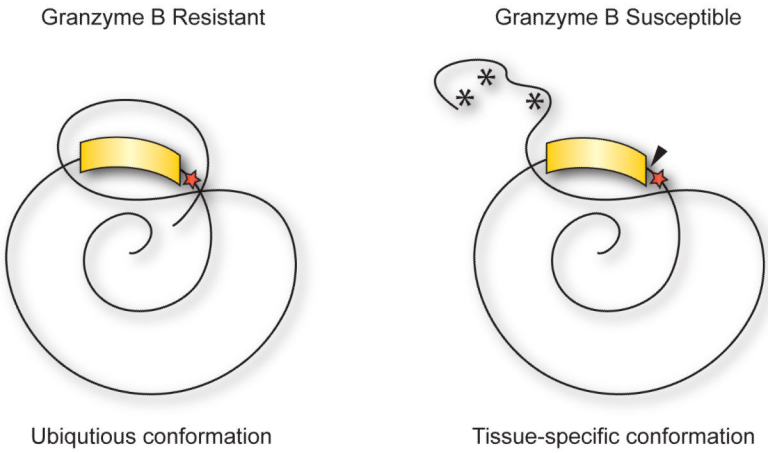

Figure 2. Cleavage of GrB-sensitive forms of autoantigens may reveal cryptic epitopes in disease relevant target tissue

GrB-sensitive forms of autoantigens may be present disease-relevant target tissue due to unique intermolecular interactions or post-translational modifications (*) exposing GrB cleavage sites (red star). Seemingly ubiquitously expressed autoantigens may be susceptible to GrB cleavage (black triangle) only when in tissue-specific immunogenic conformations and may drive the generation of autoimmunity to cryptic epitopes (yellow).

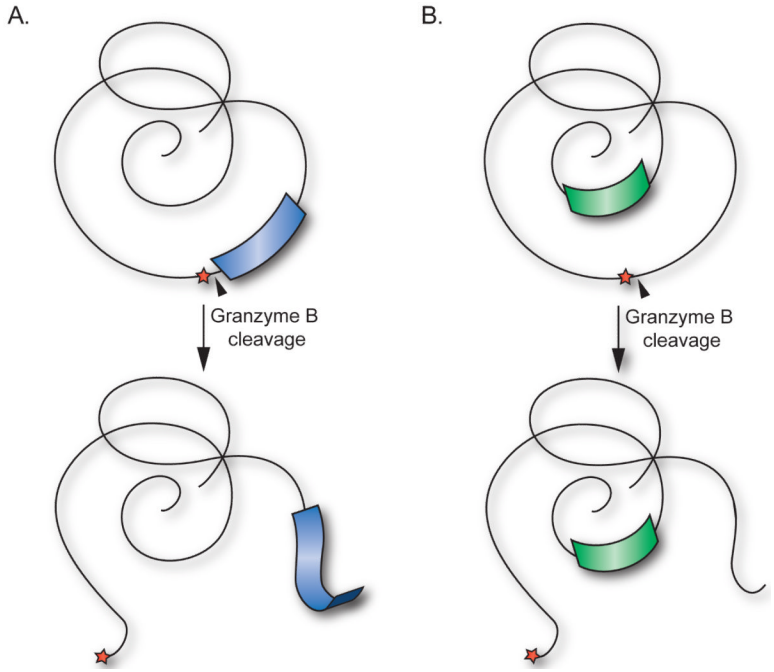

Figure 3. Cleavage of autoantigens at GrB cleavage sites located in unstructured loops may liberate cryptic epitopes derived from structural elements

(A) Proteolysis by GrB (black triangle) occurring in unstructured loops or linker regions of autoantigens (red star), may enhance presentation of cryptic epitopes derived from adjacent structural elements (blue). (B) GrB cleavage may also induce structural changes leading to increased presentation of cryptic epitopes derived from previously hidden regions of autoantigens (green).

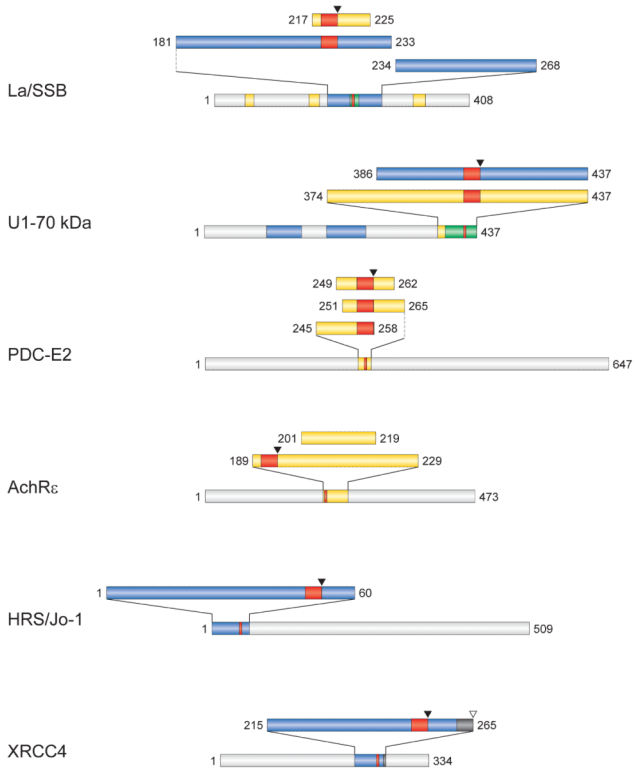

Figure 4. Autoimmune epitopes are co-located with GrB cleavage sites

B (blue) and T (yellow) cell epitopes of several autoantigens are clustered in regions containing GrB cleavage sites (red). Overlapping B and T cell epitopes (green) in La and U1-70kDa suggest autoimmune “hot spots” in these regions of antigens containing GrB cleavage sites.

Table 1

Autoantigens cleaved by granzyme B have diverse functions and subcellular localizations

Autoantigen	Accession No.	Disease Association	Function	Localization	References
acetylcholine receptor e subunit (AchRε)	NP_000071	MG	acetylcholine receptor	plasma membrane	(29)
β-Actin	NP_001092	MCB	structural protein	cytosol	(30)
AHNAK	NP_001611	SLE	calcium signalling	cytosol/nucleus	(75)
alanyl tRNA synthetase (ARS)	NP_001596	Myositis	translation	cytosol	(21)
centromere protein B (CENP-B)	NP_001801	SSc	mitosis	nucleus	(21), (67)
centromere protein C(CENP-C)	NP_001803	SSc	mitosis	nucleus	(67)
chromodomain helicase DNA binding 4 (/CHD4/Mi-2)	NP_001264	Myositis	gene expression	nucleus	(21)
DNA-PK catalytic subunit (DNA-PKcs)	NP_008835	Myositis	DNA repair	nucleus	(21), (26)
fibrillarin	NP_001427	SSc	rRNA processing	nucleolous	(21)
α-fodrin	NP_001123910	SS	cytoskeletal protein	cytosol	(25)
glutamate receptor subunit 3 (GluR3)	NP_000819	RE	glutamate receptor	plasma membrane	(24)
human endogenous retrovirus K-10 gag (HERV-K10)	P87889	SLE	endogenous retrovirus	nucleus	(76)
histidyl tRNA synthetase (HRS/Jo-1)	NP_002100	Myositis	translation	cytosol	(21), (46)
Ki-67	NP_002408	Myositis	proliferation	nucleus	(21)
Ku-70	NP_001460	Myositis, SLE	DNA repair	nucleus	(21)
La/SSB	NP_003133	SLE, SS	RNA binding	nucleus	(21)
lamin B	NP_005564	Hepatitis, SLE	structural protein	nuclear membrane	(6)
muscarinic acetylcholine receptor 3 (M3R)	NP_000731	SS	acetylcholine receptor	plasma membrane	(25)
nuclear mitotic apparatus protein 1 (NuMA)	NP_006176	SS	mitosis	nucleus	(21)
nucleolus organizing region 90kDa (NOR-90/UBF)	NP_055048	SSc	transcription factor	nucleolous	(21)
nucleophosmin (B23)	NP_002511	SSc	rRNA processing	nucleolous	(48), (50)
pyruvate dehydrogenase complex E2 (PDC-E2)	NP_001922	PBC	acetyl CoA synthesis	mitochondria	(70)
PMScl/EXOSC10	NP_001001998	Myositis/SSc overlap	mRNA degradation	cytosol	(21)
poly(ADP)ribose polymerase 1 (PARP1)	NP_001609	SLE	ribosylation	nucleus	(21)
postmeiotic segregation 1 (PMS1)	NP_000525	Myositis	DNA mismatch repair	nucleus	(21)
postmeiotic segregation 2 (PMS2)	NP_000526	Myositis	DNA mismatch repair	nucleus	(21)
RNA polymerase I (RNA Pol I)	NP_056240	SSc	transcription	nucleus	(21)
RNA polymerase II (RNA Pol II)	NP_000928	SSc	transcription	nucleus	(21)
signal recognition particle 72 kDa (SRP-72)	NP_008878	Myositis, SLE	translation	cytosol	(21)

Autoantigen	Accession No.	Disease Association	Function	Localization	References
topoisomerase 1 (Topo-1)	NP_003277	SSc	transcription	nucleus	(21)
U1 small nuclear ribonucleoprotein 70kDa (U1-70kDa)	NP_003080	Myositis, SLE, SSc	RNA processing	nucleus	(21)
ubiquitin fusion degradation 2 (UFD2)	NP_001099032	SSc	ubiquitination	nucleus	(77)
XRCC4	NP 072044	SLE	DNA repair	nucleus	(78)

Abbreviations: MCB, medullary carcinoma of the breast; MG, myasthenia gravis; PBC primary biliary cirrhosis; RE, Rasmussen's encephalitis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, scleroderma

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