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The immune response to alfaB-crystallin: self, non-self and phosphorylation

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Tolerance controls encephalitogenicity of αB-crystallin in the Lewis rat

2.

Summary

The myelin-associated stress protein αB-crystallin is a potent immunogen to human T cells. The protein is absent from normal human lymphoid tissues but it can be induced and presented via HLA-DR following viral infection. For these reasons, αB-crystallin is considered as a candidate autoantigen in multiple sclerosis. In the present study, we examined the potential of αB-crystallin to induce experimental autoimmune encephalomyelitis (EAE) in Lewis rats. Attempts to induce EAE with either bovine, rat or murine αB-crystallin or αB-crystallin peptides consistently failed. None of the protocols tested led to the development of histological or clinical signs of EAE.
Examination of lymphoid tissues of the Lewis rat revealed constitutive expression of αB-crystallin in thymus, spleen, and peripheral lymphocytes at readily detectable levels, in sharp contrast to the situation in humans. Accordingly, Lewis rat T cells were found to be fully tolerant to homologous rat αB-crystallin. Yet, they did develop marked T-cell responses to heterologous bovine αB-crystallin. T cells raised against the bovine protein responded to a part of the bovine sequence that contained three out of the five amino acid substitutions between bovine and rat αB-crystallin and they failed to recognize the corresponding rat sequence. These data show that in Lewis rats, constitutive lymphoid expression of αB-crystallin results in a state of profound nonresponsiveness to homologous αB-crystallin sequences, effectively controlling the development of EAE in response to this myelin antigen.

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Introduction

The small stress protein αB-crystallin was previously identified as an immunodominant myelin protein to human T cells, eliciting pro-inflammatory cytokine production (1). Also, αB-crystallin was found to be expressed at strongly elevated levels in astrocytes and oligodendrocytes in affected central nervous system tissue of multiple sclerosis (MS) patients (2). Expression of αB-crystallin in the presence of co-stimulatory factors in central nervous system myelin of MS patients may render αB-crystallin a target for αB-crystallin-specific T cells, resulting in autoimmune demyelination.
αB-Crystallin is an evolutionary highly conserved protein of approximately 20 kDa and a member of the family of small heat shock proteins (3). Besides serving as a structural component of the eye lens, αB-crystallin is expressed in several other tissues, like the heart, skeletal muscle and kidney epithelium (4). Moreover, expression and post-translational phosphorylation of αB-crystallin can be induced by various conditions of cellular stress (5-7).
The present study was aimed at investigating the capacity of αB-crystallin or peptides derived from it, to induce demyelinating autoimmune disease in Lewis rats. The Lewis rat was used because it is known to be susceptible to many T cell-mediated, organ specific autoimmune diseases such as experimental allergic encephalomyelitis (EAE) (8, 9), arthritis (10, 11), and experimental autoimmune uveoretinitis (12). The autoimmune condition can be elicited in Lewis rats by immunization with protein or synthetic peptides in adjuvant, or by adoptive transfer of activated antigen specific T cells. Many different myelin components, such as myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein, and S100β, have been shown to be encephalitogenic in Lewis rats (reviewed by Martin and McFarland, 13).
In this report we show that Lewis rats are resistant to EAE induction with αB-crystallin or peptides thereof, despite the presence of the protein in central nervous system myelin. It appeared that Lewis rat T cells are tolerant to rat and murine αB-crystallin, but not to bovine αB-crystallin. PCR analysis and Western blotting revealed constitutive expression of αB-crystallin in primary and secondary lymphoid tissues. This situation is associated with a state of T cell tolerance, and clearly differs from what is observed in humans, where αB-crystallin is undetectable in healthy lymphoid cells (14).

Materials and Methods

Rats
Male Lewis/CrlBr rats were obtained from Charles River/the Broekman Institute, Someren, the Netherlands.

Antigens
αB-Crystallin was purified from bovine, rat, and murine eye lenses using size exclusion chromatography and reversed-phase HPLC as described (15). The amino acid sequences of rat, murine, and bovine αB-crystallin are given in Table 1.
Partially overlapping 16-mer peptides spanning the complete murine αB-crystallin sequence were made on an Abimed 422 robotic multiple synthesizer as peptide amides (CONH2) as previously described (16).

```
                   10        20        30        40        50
rat       MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFSTATSLSPFYLR
murine    --------------------------------------------------
bovine    --------------------------------------PAS---------

                   60        70        80        90       100
rat       PPSFLRAPSWIDTGLSEMRMEKDRFSVNLDVKHFSPEELKVKVLGDVIEV
murine    -------------------L------------------------------
bovine    -------------------L------------------------------

                  110       120       130       140       150
rat       HGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRK
murine    --------------------------------------------------
bovine    -------------------------------A------------------

                  160       170
rat       QASGPERTIPITREEKPAVTAAPKK
murine    -V-----------------A-----
bovine    -------------------------
```

Table 1. Amino acid sequences of rat, murine, and bovine αB-crystallin.

Immunizations
Different immunization protocols, listed in Table 2, were tested to attempt to induce EAE. Rats were immunized s.c. in both hind feet with 100 μl of an emulsion containing 50-200 μg antigen in CFA (2 mg/ml Mycobacterium tuberculosis H37 RA, Difco Laboratories). Some rats were boosted i.p. on day 15

with an emulsion of the same antigen in IFA. One group of rats was immunized i.p., on day 0 and day 15, with 300 μl of an emulsion containing 25 μg of each murine αB-crystallin peptide and CFA (day 0) or IFA (day 15). Another group of rats was immunized s.c. with an 1:1 emulsion of 200 μg αB-crystallin in 20 mg/ml dimethyldioctadecylammonium bromide (DDA) (Phase Separations).

Lymphocyte proliferation assay

Proliferative responses of lymphocytes were measured in flat-bottom 96 well microtiter plates in triplicate cultures in a final volume of 0.2 ml culture medium: Iscove's modified DMEM with Glutamax (Gibco BRL), supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5 x 10^{-5} M 2-ME, and 10% (vol/vol) fetal calf serum. Splenocytes or lymph node cells were seeded at 2 x 10^{5} per well in the presence of varying doses of antigen. After 72 h of culture, 20 kBq [^{3}H]-thymidine was added to the cultures and after another 18 h, cells were harvested and [^{3}H]-thymidine incorporation was determined using a β-plate counter (Canberra Packard). Proliferative responses are expressed as stimulation indices.

RT-PCR

Single cell suspensions of thymus, spleen and cardiac muscle were prepared. PBMC and splenocytes were isolated by density gradient centrifugation using Lympholyte-M (Cedar Lane). Cells were washed twice with PBS, frozen in liquid nitrogen and stored at -70°C until RNA isolation was performed. mRNA was isolated using RNAzol™ B method (Campro Scientific) and isopropanol precipitation. Using 1 μg of mRNA as a template, cDNA was produced using the Reverse Transcription System (Promega). For amplification, cDNA (3 μl) was added to 1 μl 10 mM dNTP-mix, 5 μl 10x Taq polymerase buffer (500 mM KCl, 600 μg/ml BSA, 100 mM Tris-HCl pH 8.0, 30 mM $MgCl_2$), and 1 U Taq polymerase (Life Technologies) in a final volume of 50 μl. αB-Crystallin specific primers were 5' TGCRGTGACAGCAGGCTTCT 3' and 5' GAGAGCACCTGTT GGAGTCT 3'. Reaction conditions included pre-melting at 94°C for 4 minutes followed by 40 cycles of melting at 94°C, annealing at 65°C and extension at 72°C for 30 s each. β-Actin encoding mRNA amplification was performed for all samples to verify adequate cDNA synthesis.

Myelin isolation

Myelin proteins were isolated from Lewis rat brain and fractionated by reversed phase-HPLC as described (17). Briefly, myelin proteins were isolated by density-gradient centrifugation, delipidated and fractionated by reversed phase HPLC. Myelin fractions were lyophilized and resuspended in equal volumes of water.

Standard SDS-PAGE analysis of the fractions was performed using a 8-25% gradient polyacrylamide gel (Pharmacia LKB). Western blots were prepared and expression of αB-crystallin in the fractions was detected using a mouse monoclonal antibody JAM01 that was raised against bovine αB-crystallin.

Western blot analysis
Thymus, spleen and cardiac muscle were homogenized in 8 M ureum, 20% (vol/vol) acetic acid using a Dounce homogenizer. Soluble proteins were separated on a C4-reversed phase HPLC column. Proteins that eluted at 31% to 44% (vol/vol) acetonitrile in an aqueous solvent containing 0.1% (vol/vol) trifluoroacetic acid were collected and lyophilized. Protein samples were dissolved in water and subjected to standard SDS-PAGE analysis using a 8-25% gradient polyacrylamide gel (Pharmacia LKB). Western blots were prepared and expression of αB-crystallin in the tissue samples was detected using polyclonal rabbit antibodies raised against human myelin αB-crystallin (1).

Delayed Type Hypersensitivity (DTH) measurement
DTH reactions were measured 24 hr and 48 hr after injection of 100 μg protein (in 50 μl PBS) in the ear, using a pressure-sensitive micrometer (Mitutoyo). The contra-lateral ear was injected with 50 μl PBS alone and served as a control. Values are expressed as (mm $ear_{protein}$ - mm ear_{PBS}) with standard deviations.

Results

Characterization of the Lewis rat T cell response to αB-crystallin
αB-Crystallin is an evolutionary highly conserved protein. We started our studies using bovine αB-crystallin and a set of 16-mer peptides (8 amino acids overlapping) spanning the entire murine αB-crystallin sequence were available. As indicated in Table 1, the amino acid sequence of rat αB-crystallin differs at 3 positions from the murine sequence, and at 5 positions from the bovine sequence. To characterize the T cell response to αB-crystallin, Lewis rats were immunized with bovine αB-crystallin. Lymphocytes of these rats strongly proliferated in response to the protein, but no specific proliferation was measured in response to any of the peptides tested (data not shown). In a parallel experiment, rats were immunized with a mixture of all the peptides. As can be seen in Figure 1, splenocytes of these rats showed a dominant proliferative response to peptide 49-64. The discrepancy between responses to determinant 49-64 after priming with either murine peptides or bovine protein, can not be explained by amino acid

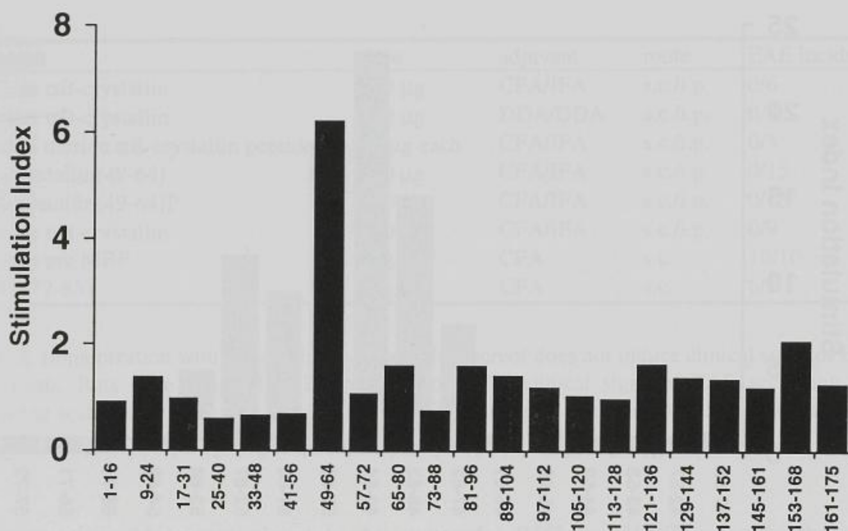

Figure 1. T cell epitopes of αB-crystallin. Lewis rats were immunized with a mixture of synthetic peptides corresponding to the murine αB-crystallin sequence. Proliferative responses of splenocytes to 25 μg/ml of the individual peptides were determined in a proliferation assay.

substitutions between the species, since 49-64 is a sequence that is identical between the bovine, murine and rat homologues. Lack of reactivity to epitope 49-64 after priming with bovine αB-crystallin therefore indicates that epitope 49-64 is probably not efficiently processed out of the entire protein and presented to T cells, i.e. 49-64 is a cryptic epitope [18-20]. The observed response to bovine αB-crystallin is species-specific, as will be discussed below.

To determine the core of the dominant epitope contained within peptide 49-64, we immunized rats with peptide 49-64 and tested T cell proliferative responses to a series of peptides ranging from 41-56 to 57-72. Strong responses were measured to peptides ranging from 47-62 to 53-68, indicating that the core of the epitope is sequence 53-62 (Figure 2). This sequence fits well with both published MHC binding motifs for RT1.Bl [21, 22], suggesting that Asp_{62} represents the negatively charged major MHC anchor residue at relative position 9.

It has been reported for SJL mice that T cells discriminate between differentially phosphorylated forms of αB-crystallin [23]. Since one of the known phosphorylation sites of αB-crystallin, Ser_{59} [24], is contained within epitope 53-62, we tested whether Lewis rat T cells specifically recognize this phosphorylated residue. It appeared that Lewis rat lymphocytes are cross-reactive to differentially

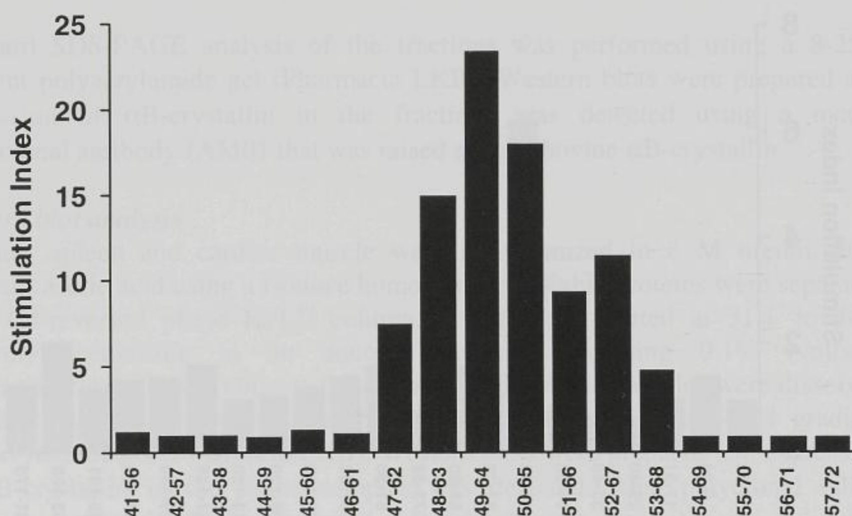

Figure 2. Delineation of the core region of T-cell epitope 49-64. Lewis rats were immunized with 49-64/CFA and proliferative responses of lymph node cells to 25 μg/ml of overlapping peptides were determined.

phosphorylated forms of αB-crystallin, both at the level of protein specific responses, as well as at the level of responses to epitope 49-64 (data not shown). T cells responded stronger to unphosphorylated 49-64 than to phosphorylated 49-64, irrespective of which peptide was used for priming. This suggests that phosphorylation of Ser_{59} reduces binding of the determinant to the MHC molecule. Indeed, the RT1.Bl binding motif described by Reizis predicts Ser_{59} to be a major MHC anchor residue at position 6.

Lewis rats are resistant to EAE induction with αB-crystallin, despite its presence in the central nervous system

We next tested whether αB-crystallin could induce demyelinating autoimmune disease in Lewis rats. Therefore, Lewis rats were immunized with bovine αB-crystallin or with synthetic peptides of the cryptic T cell epitope 49-64 in its unphosphorylated or phosphorylated state, using CFA as an adjuvant. None of the rats tested showed signs of paralysis or weight loss (Table 2). Also other protocols for immunization with αB-crystallin (listed in Table 2) did not result in clinical signs of EAE. Histological examination of brains and spinal cords of animals that were immunized with αB-crystallin or peptides thereof also revealed no signs of

Antigen	dose	adjuvant	route	EAE incidence
Bovine αB-crystallin	200 μg	CFA/IFA	s.c./i.p.	0/6
Bovine αB-crystallin	200 μg	DDA/DDA	s.c./i.p.	0/3
Pooled murine αB-crystallin peptides	25 μg each	CFA/IFA	s.c./i.p.	0/3
αB-crystallin(49-64)	100 μg	CFA/IFA	s.c./i.p.	0/15
αB-crystallin(49-64)P	100 μg	CFA/IFA	s.c./i.p.	0/15
Bovine αB-crystallin	100 μg	CFA/IFA	s.c./i.p.	0/9
Guinea pig MBP	50 μg	CFA	s.c.	10/10
MBP(72-85)	50 μg	CFA	s.c.	6/7

Table 2. Immunization with αB-crystallin or peptides thereof does not induce clinical signs of EAE in Lewis rats. Rats were weighed daily and monitored for clinical signs of EAE according to the following scale: 0, no signs; 0.5, tail paresis; 1, tail paralysis; 2, paraparesis; 2.5, partial paralysis; 3, complete paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death.

inflammation (data not shown). As controls, 85% to 100% of rats that were immunized with MBP72-85 or guinea pig MBP developed clinical signs of EAE within 13 days after immunization.

A prerequisite for organ-specific autoimmunity is the expression of the antigen in the target organ. Therefore, to determine whether αB-crystallin is present in rat CNS myelin, we isolated myelin from healthy rat brain by sucrose gradient purification [25]. Myelin proteins were delipidated and fractionated by reversed phase-HPLC [17]. Western blot analysis of the individual myelin fractions revealed that αB-crystallin is indeed present as target antigen at readily detectable levels in rat CNS myelin (Figure 3).

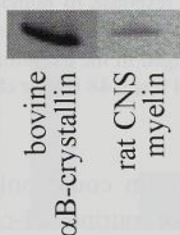

Figure 3. αB-Crystallin is expressed in rat central nervous system myelin. Myelin proteins were isolated from brain tissue from healthy rats and fractionated by reversed phase-HPLC. Western blot analysis of the fractions was performed using an αB-crystallin-specific antibody. Bovine αB-crystallin was used as a positive control.

Lewis rats are tolerant to rat and murineαB-crystallin, but not to bovine αB-crystallin

One explanation for the observed resistance to EAE is that the T cells that are activated by the described immunizations using bovine or murine material do not recognize αB-crystallin as it is present in the rat central nervous system. Bovine αB-crystallin that was used for the immunizations differs in 5 out of the 175 amino acids and the synthetic peptides may correspond to determinants that are not naturally processed out of the entire protein.

To test whether there are any differences between T cell responses to homologous versus heterologous αB-crystallin, rats were immunized with bovine, rat, or murine αB-crystallin. Whereas rats immunized with bovine αB-crystallin showed strong delayed type hypersensitivity (DTH) reactions to this protein, rats immunized with rat or murine αB-crystallin showed only minor reactivity (Figure 4).

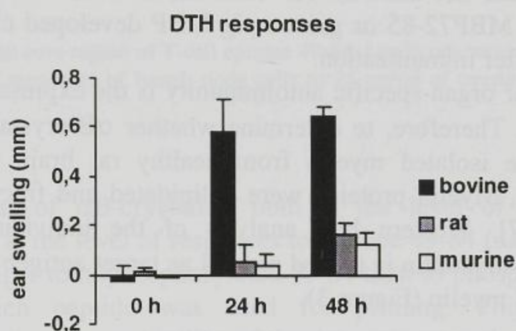

Figure 4: Lewis rats do not show a DTH response to homologous αB-crystallin. Nine days after immunization with bovine (black bars), rat αB-crystallin (hatched bars), or murine αB-crystallin (white bars) αB-crystallin, rats were challenged in the ear with 100 μg of the corresponding protein. Ear swelling (in mm + SD) was measured 24 h and 48 h after challenge.

Also, in vitro lymphocyte proliferation could only be measured in response to bovine αB-crystallin, but not to rat or murine αB-crystallin (Figure 5). In repeated experiments, low T cell responsiveness to rat and murine αB-crystallin was sometimes observed, indicating that T cell tolerance to homologous αB-crystallin is not complete. Thus, despite minimal differences in sequence between bovine, rat and murine αB-crystallin, distinct differences exist in T cell reactivity to these homologues in rats. As expected by the lack of proliferation and DTH responses,

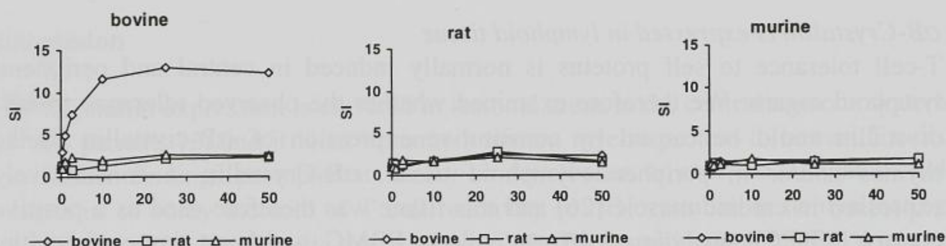

Figure 5. Lewis rats are tolerant to rat and murine αB-crystallin, but not to bovine αB-crystallin. Rats were immunized with bovine αB-crystallin (left panel), rat αB-crystallin (middle panel), or murine αB-crystallin (right panel). Eleven days after immunization, proliferative responses of lymph node cells to increasing concentrations of bovine αB-crystallin (diamonds), rat αB-crystallin (squares), or murine αB-crystallin (triangles) were determined.

rat αB-crystallin did not induce any histological or clinical signs of EAE in Lewis rats (data not shown).

Since 3 out of the 5 substitutions between bovine and rat αB-crystallin are clustered at positions 39, 40, and 41, we examined T cell responses to this particular sequence as a potential epitope explaining species-specific responses. Lymphocytes that were primed with bovine αB-crystallin responded well to synthetic peptide 31-50 representing the bovine sequence, but did not respond to the peptide representing the rat sequence (Figure 6). Thus, T cells that are specific for conserved self sequences of αB-crystallin are purged from the Lewis rat T cell repertoire, whereas T cells to bovine-specific sequences persist.

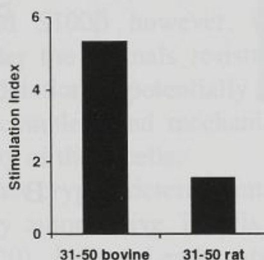

Figure 6. Lymphocytes only respond to a peptide corresponding to the bovine αB-crystallin sequence. Proliferation of lymph node cells that were raised against bovine αB-crystallin was measured in response to peptides representing either the bovine or the rat sequence 31-50.

αB-Crystallin is expressed in lymphoid tissue

T-cell tolerance to self proteins is normally induced in central and peripheral lymphoid organs. We therefore examined whether the observed tolerance to αB-crystallin could be caused by constitutive expression of αB-crystallin in the thymus and/or in peripheral lymphoid tissue. αB-Crystallin is constitutively expressed in cardiac muscle [26] and this tissue was therefore used as a positive control. RT-PCR analysis of thymus, spleen, PBMC, and heart tissue of healthy Lewis rats using αB-crystallin specific primers showed that αB-crystallin-encoding mRNA is readily detectable in all tissues (Figure 7A). To confirm expression of αB-crystallin, total protein extracts from thymus, spleen, and heart were isolated and separated by SDS-PAGE. Western blot analysis clearly showed the presence of αB-crystallin in heart and thymus and, albeit at somewhat lower levels, in the spleen (Figure 7B).

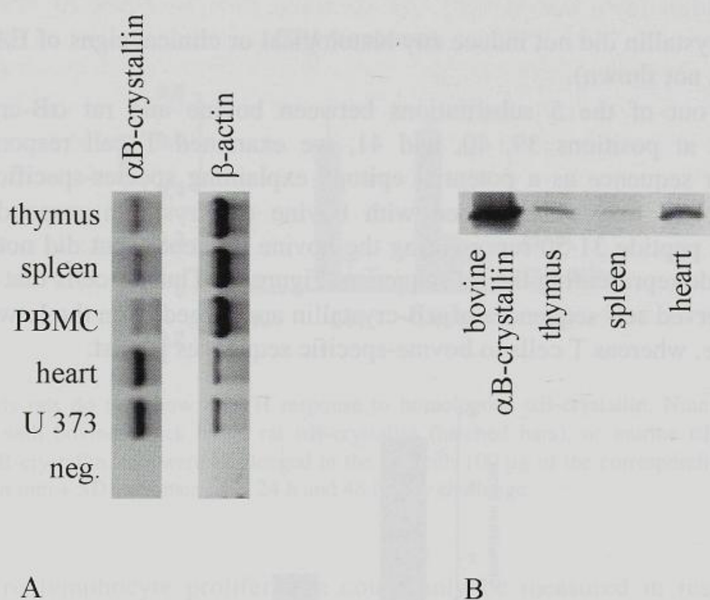

Figure 7. αB-Crystallin is expressed in lymphoid organs of the Lewis rat. (A) RT-PCR was performed using αB-crystallin-specific primers on mRNA isolated from PBMC, spleen, thymus and, as a positive control, cardiac muscle. (B) αB-Crystallin protein expression was verified by Western blot analysis of soluble proteins. Bovine αB-crystallin was used as a positive control.

Discussion

αB-Crystallin expression is elevated in lesional areas in the central nervous system of MS patients (2) and it is an immunodominant myelin protein to human T cells (1). These findings prompted us to try to develop an animal model that would enable us to study the role of T cell reactivity to αB-crystallin in demyelinating autoimmune disease. Despite the expression of αB-crystallin in rat central nervous system tissue and despite the presence of a strong T cell response to bovine αB-crystallin, Lewis rats were resistant to EAE induction either by whole bovine protein or by peptides derived from murine αB-crystallin. It appeared that Lewis rats express αB-crystallin in lymphoid tissues and are tolerant to homologous αB-crystallin. This is in sharp contrast to the situation in humans, where αB-crystallin is not expressed in healthy lymphoid tissue and peripheral T cells respond to self αB-crystallin in a pro-inflammatory way (1, 14). Considering the presence of αB-crystallin-encoding mRNA in lymphoid organs of most species (14), including Lewis rats, it is likely that the strong T cell reactivity towards αB-crystallin as it is observed in humans is an exception among mammals. Rather, T cell tolerance, as we have now shown for the Lewis rat, is to be expected.

It has been shown that susceptibility to EAE is related to the ability to develop a Th1 type response to a myelin antigen (27). The firm state of T cell tolerance to self αB-crystallin, as determined by the absence of proliferation and Th1 (DTH) responses, renders Lewis rats resistant to EAE induction with this protein. Our results are in accordance with the finding that thymic expression of retinal autoantigens correlates with resistance to autoimmune uveitis (28). Also, experimental introduction of autoantigen into the thymus has proven effective in preventing autoimmune disease (29). For the central nervous system proteins myelin basic protein and S100β however, it has been shown that thymic expression does not render the animals resistant to EAE induction with these proteins (30, 31). Clonal deletion of potentially autoreactive T cells in the thymus is obviously not always complete and mechanisms of peripheral tolerance may operate to control reactivity of these cells.

It is generally accepted that cryptic determinants can escape tolerance induction, and can be recognized by autoreactive T cells under particular conditions, e.g. altered processing (18-20). Indeed Lewis rats are not tolerant to the cryptic determinant 49-64 and can mount a Th1 type response to the peptide, as measured by a strong DTH reaction (data not shown). Nevertheless, determinant 49-64 does not induce EAE. It is thus very likely that the inability to process determinant 49-64 out of the entire protein is the mechanism that precludes T cell reactivity to αB-crystallin in vivo in Lewis rats that were primed with 49-64.

Possibly, expression of αB-crystallin in the thymus may also lead to an irreversible state of tolerance for most potential self epitopes by means of clonal deletion. In Biozzi mice an αB-crystallin sequence induces clinical signs of EAE at low incidence (S. Amor, personal communication). The incompleteness of T cell tolerance in this particular strain of mice may be the consequence of lack of expression of αB-crystallin-encoding mRNA in the thymus of these mice (14).
The data described in this paper show that Lewis rat T cells are tolerant to self αB-crystallin. Mechanisms of central as well as peripheral tolerance induction may operate to control the αB-crystallin specific cells. It has been suggested by Van Eden and colleagues (32) that T cells that are reactive with self heat shock protein 60 are of a regulatory type and thereby confer resistance to autoimmune diseases like arthritis in humans and in rats (33, 34). We have no indications for an analogous regulatory role of αB-crystallin-specific reactivity. That is, no T cell responses to self αB-crystallin were detectable in rats. And notably, T cell responses to human αB-crystallin in humans are of a pro-inflammatory, i.e. IFN-γ producing, potentially auto-aggressive type (1). Thus this paper illustrates that striking differences exist in the way heat shock proteins are expressed in mammals, and in the way the immune system deals with them.

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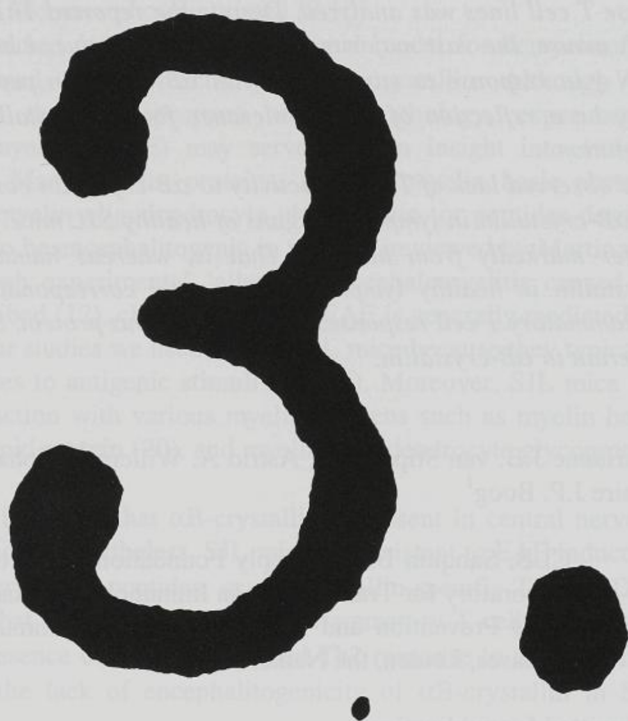
Functional tolerance controls encephalitogenicity of αB-crystallin in SJL mice



Summary

αB-Crystallin is considered a candidate autoantigen in multiple sclerosis. In this study we examined the capacity of αB-crystallin to induce experimental autoimmune encephalomyelitis (EAE) in C57Bl/6, Balb/c, C3H, SWR, PL/J, and SJL mice. Despite extensive efforts to induce EAE using immunizations with αB-crystallin or peptides thereof, or using adoptive transfer of αB-crystallin specific lymphocytes, no clinical or histological signs of EAE were observed.
Immunization of SJL mice with whole bovine αB-crystallin resulted in marginal proliferative responses. After immunization with murine αB-crystallin however, no proliferative or delayed type hypersensitivity response could be detected at all. This indicates that the cellular response to murine αB-crystallin is under tight control. Nevertheless, a large number of αB-crystallin determinant-specific T cell lines could be raised following immunization with peptides representing the immunodominant T cell epitope 41-56 of αB-crystallin. The cytokine profile of these T cell lines was analyzed. Despite the reported Th1-favouring nature of the SJL mouse, the vast majority of T cell lines produced high levels of IL-4 but no IFN-γ in response to stimulation with αB-crystallin peptide 41-56. This finding may be a reflection of T cell tolerance for αB-crystallin by means of immune deviation.
The observed lack of T cell reactivity to αB-crystallin corresponded to expression of αB-crystallin in lymphoid organs of healthy SJL mice. In this regard, SJL mice differ markedly from humans. That is, whereas humans do not express αB-crystallin in healthy lymphoid tissues and correspondingly mount strong pro-inflammatory T cell responses to the autologous protein, SJL mice are functionally tolerant to αB-crystallin.

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Introduction

Recent data indicate that the myelin-associated stress protein αB-crystallin can play a key role in the autoimmune pathology of multiple sclerosis (MS). αB-Crystallin is expressed in astrocytes and oligodendrocytes in multiple sclerosis lesions (1) and it is an immunodominant myelin antigen to human T cells, triggering significant IFN-γ production (2). Also, virally infected human B cells can express αB-crystallin and stimulate αB-crystallin specific T cells (3). Activation of αB-crystallin specific T cells and release of pro-inflammatory cytokines in the central nervous system may contribute to the pathogenesis of MS.
αB-Crystallin is a protein of 20 kD that is constitutively expressed in several organs such as the eye lens and cardiac muscle (4). Based on its chaperone properties and sequence homology with hsp25, αB-crystallin has been classified as a small heat shock protein (5, 6). Expression of αB-crystallin in glia cells can be upregulated by various conditions of cellular stress (1, 7, 8). Under such conditions, αB-crystallin becomes phosphorylated (8-10).
The current study was aimed at developing an animal model for demyelinating autoimmune disease based on autoreactivity to αB-crystallin. Although not necessarily representing the full spectrum of MS pathology, experimental autoimmune encephalomyelitis (EAE) may serve to gain insight into various aspects of the disease. Many myelin proteins, such as myelin basic protein, proteolipid protein and myelin oligodendrocyte glycoprotein, or peptides derived from them have proven to be encephalitogenic in rodents (reviewed by Martin and McFarland, 11). Although experimental 'allergic' encephalomyelitis caused by Th2 cells has been described (12), classical MS-like EAE is generally mediated by Th1 cells (13-15). For our studies we used female SJL mice because they typically mount Th1 type responses to antigenic stimuli (16, 17). Moreover, SJL mice are susceptible to EAE induction with various myelin antigens such as myelin basic protein (18, 19), proteolipid protein (20), and myelin oligodendrocyte glycoprotein (21).
In the present report, it is shown that αB-crystallin is present in central nervous system myelin of SJL mice. Nevertheless, SJL mice are resistant to EAE induction with αB-crystallin, αB-crystallin peptides, or αB-crystallin-specific T cells. Data presented here suggest that the absence of a pro-inflammatory T cell response to αB-crystallin and the presence of a strongly biased Th2 response to αB-crystallin sequences account for the lack of encephalitogenicity of αB-crystallin in SJL mice.

Materials and Methods

Mice

Female C3H, SWR, and PL/J mice were obtained from Charles River/the Broekman Institute, Someren, the Netherlands. Female C57Bl/6 and Balb/c mice were obtained from TNO Prevention and Health, Leiden, the Netherlands. Female SJL ($H\text{-}2^s$) mice were obtained from the Erasmus University, Rotterdam, the Netherlands. All mice were used between 8 and 12 weeks of age.

Antigens

Synthetic peptides corresponding to the murine αB-crystallin sequence 41-56 (ATSLSPFYLRPPSFLR) were obtained from Genosys Biotechnologies (Cambridge, UK). The natural site of phosphorylation in this sequence is serine at amino acid position 45. The synthetic peptide containing a phosphate group at Ser_{45} is abbreviated as 41-56P. Peptide $PLP_{139\text{-}151}$ (HCLGKWLGHPDKF) corresponding to the rat proteolipid sequence 139-151 was synthesized at TNO Prevention and Health (Leiden, the Netherlands). αB-Crystallin was isolated from bovine or murine eye lenses by size exclusion chromatography and reversed phase-HPLC as previously described (22). Myelin basic protein was isolated from spinal cord tissue from guinea pigs using the method described by Eylar et al (23).

Immunizations

Naive SJL mice were immunized s.c. with 100-400 µg αB-crystallin or peptides derived from it emulsified in CFA (containing 1-5 mg/ml Mycobacterium tuberculosis, Difco Laboratories, Detroit, MI). On the day of immunization and 48 hr later, mice were injected i.v. with 200 ng pertussis toxin from Bordetella pertussis (Speywood Pharmaceuticals, Maidenhead, UK). Some groups of mice received a second identical set of immunizations 7 days after the first immunization. As a positive control, EAE was induced in naive SJL mice by s.c. immunization with 50 µg $PLP_{139\text{-}151}$ emulsified in CFA (containing 1 mg/ml Mycobaterium tuberculosis). On the day of immunization and 48 hr later, mice were injected i.v. with 200 ng pertussis toxin from Bordetella pertussis (Speywood Pharmaceuticals, Maidenhead, UK).

Generation of T cell lines

At day 0 and at day 7 mice were immunized s.c. at 2 sites in the flanks with an emulsion of 200 µg αB-crystallin peptide in complete Freund's adjuvant containing 5 mg/ml Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). At day 21 after immunization, lymph node cells were isolated and

restimulated in vitro with either 5 µg/ml peptide 41-56 (for cell lines O) or 5 µg/ml peptide 41-56P (for cell lines P) in culture medium (RPMI1640, Dutch modification; supplemented with 100 U ml/ml penicillin, 0.1 mg ml/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 x 10^{-5} M 2-ME) with 10% (vol/vol) FCS. After 4 days of culture, dead cells were removed using a Lympholyte-M (Cedar Lane, Hornby, Ontario, Canada) gradient. Viable cells were propagated in culture medium supplemented with 10% (vol/vol) FCS, IL-2 and 1% (vol/vol) non-essential amino acid solution (Flow Laboratories, Irvine, UK). After 7 days, lymphocytes were seeded at 2,000 cells/well or 20,000 cells/well in round-bottomed 96 well plates. These cell lines were cyclically restimulated for 3 to 4 days in the presence of 2 x 10^5 irradiated (30 Gy) syngeneic splenocytes and thymocytes as antigen presenting cells (APC) per well and peptide (see above) and then propagated for 7 days in the presence of IL-2. Antigen specific cell lines (stimulation index greater than 2) were identified using a split well assay. Cell lines were cultured for 65 to 88 days before proliferation and cytokine production were analyzed.

Proliferation assay
Proliferative responses of freshly isolated lymph node cells were measured in flat-bottomed microtiter plates in triplicate cultures in a final volume of 0.2 ml culture medium. Lymph node cells were seeded at 2 x 10^5 cells/well in the presence of 10% (vol/vol) FCS, and varying doses of antigen. Proliferative responses of cell lines were measured in round-bottomed microtiter plates in triplicate cultures in a final volume of 0.2 ml culture medium in the presence of 2 x 10^5 APC's per well, 2% (vol/vol) mouse serum, and 5 µg/ml peptide 41-56 or peptide 41-56P. After 72 h of culture, 100 µl culture supernatant per well was removed for cytokine analysis and 20 kBq [^{3}H]-thymidine was added to the cultures. After another 18 h, [^{3}H]-thymidine incorporation was determined using a ß-plate counter (Canberra Packard, Meriden, CT). Proliferative responses are expressed as stimulation indices.

Cytokine ELISA
Culture supernatants were collected 72 hr after antigenic stimulation. The concentrations of IL-4 and IFN-γ in the supernatants were determined by standard ELISA technique using Quantigen™ sets of antibodies and recombinant cytokine standard (PharMingen, San Diego, CA) according to the manufacturer's protocol.

Adoptive transfer
Mice were immunized s.c. with either 400 µg guinea pig myelin basic protein or

400 μg bovine αB-crystallin, emulsified in CFA (containing 2 mg/ml Mycobacterium tuberculosis). Eleven days after immunization, draining lymph node cells were isolated and stimulated in vitro in culture medium with 10% FCS and 25 μg/ml of the protein used for immunization. After 4 days of culture, lymphocytes were collected and washed twice with PBS. Naive recipient mice received 20-40 x 10^6 activated lymphocytes i.v.

Evaluation of EAE

Mice were weighed daily and monitored for clinical signs of EAE according to the following scale: 0, no signs; 0.5, tail paresis; 1, tail paralysis; 2, paraparesis; 2.5, partial paralysis; 3, compete paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death. Frozen sections were prepared from brain and spinal cord tissue. As a marker for microglia and macrophages, acid phosphatase activity was detected using standard histochemical techniques. Slides were counter-stained with haematoxylin and evaluated for the presence of cellular infiltrates.

Protein isolation

Eye lens, thymus, spleen, peripheral blood mononuclear cells (PBMC), brain, and cardiac muscle of healthy SJL mice were isolated and pulverized on dry ice. Brain tissue was homogenized in 80% (vol/vol) tetrahydrofurane, 1% (vol/vol) trifluoroacetic acid in water. Brain homogenate was delipidated with ether. Eyes, thymus, spleen, heart and PBMC were homogenized in an aqueous solution with 8 M ureum and 20% (vol/vol) acetic acid. Protein samples were submitted to reversed phase-HPLC using a Vydac C4 column (Chrompack Inc., Raritan, NJ) and a crude αB-crystallin preparation was collected as previously described (22). The protein samples were dialyzed extensively against water, and lyophilized.

Western blot analysis

Protein samples were dissolved in water and subjected to standard SDS-PAGE analysis using a 8-25% gradient polyacrylamide gel (Pharmacia LKB, Bromma, Sweden). Western blots were prepared and expression of αB-crystallin in the samples was detected using a mouse monoclonal antibody JAM01 that was raised against bovine eye lens αB-crystallin (2).

Results

Despite its presence in the CNS, αB-crystallin is not encephalitogenic in SJL mice

In the present study, we tried to develop an animal model for demyelinating

autoimmune disease based on autoreactivity to αB-crystallin. αB-Crystallin was purified from bovine and murine eye lenses as previously described (22). Bovine αB-crystallin is 97% homologous to murine αB-crystallin. In a first experiment we tested the encephalitogenic potential of bovine αB-crystallin in C57Bl/6, Balb/c, C3H, SWR, PL/J and SJL mice (5 mice per group) using a protocol involving two immunizations with CFA and pertussis toxin. During an observation period of two months, none of the mice developed clinical signs of EAE (data not shown).
For further studies we selected the SJL mouse, because it is known to be susceptible to EAE induction with various myelin antigens or peptides derived thereof (reviewed by Martin and McFarland, 11). We previously reported that the dominant T cell epitopes for SJL mice are 100% conserved between bovine and murine αB-crystallin (24). Both murine and bovine αB-crystallin were tested for their capacity to induce EAE in SJL mice, using the protocol described above. None of the mice tested developed clinical signs of EAE (Table 1). Central nervous system tissue of 11 mice was isolated between 7 and 21 days after immunization with bovine αB-crystallin for histochemical evaluation of signs of inflammation. No cellular infiltrates were observed in brain or spinal cord tissue of these mice (data not shown).

Treatment	EAE incidence
Immunization	
Bovine αB-crystallin	0/82
Murine αB-crystallin	0/22
αB-crystallin 1-16	0/37
αB-crystallin 41-56	0/53
αB-crystallin 41-56P	0/58
PLP 139-151	38/41
Adoptive transfer	
αB-crystallin primed lymphocytes	0/10
MBP primed lymphocytes	7/7

Table 1. Immunization with αB-crystallin or peptides thereof does not induce EAE in SJL mice.

Next, we analyzed the T cell response to murine and bovine αB-crystallin both in vitro and in vivo. Mice were immunized with bovine αB-crystallin, murine αB-crystallin, or the encephalitogenic epitope $PLP_{139-151}$ as a control. Lymphocytes of

mice that were immunized with murine αB-crystallin showed no proliferative responses to either bovine or murine αB-crystallin (Figure 1A). Bovine αB-crystallin-primed lymphocytes showed minor proliferative responses to both bovine and murine αB-crystallin (Figure 1B). In contrast, lymphocytes of mice that were primed with $PLP_{139-151}$ responded strongly to $PLP_{139-151}$ (Figure 1C). As a control, lymphocytes of all three groups of mice showed comparable proliferative responses to Mycobacterium tuberculosis, a component of the adjuvant used for immunization (data not shown). Also, we analyzed in vivo delayed type hypersensitivity responses of the immunized mice. In accordance with the in vitro data, a strong DTH response could be measured in response to $PLP_{139-151}$, but specific DTH responses to bovine and murine αB-crystallin were barely detectable (data not shown).

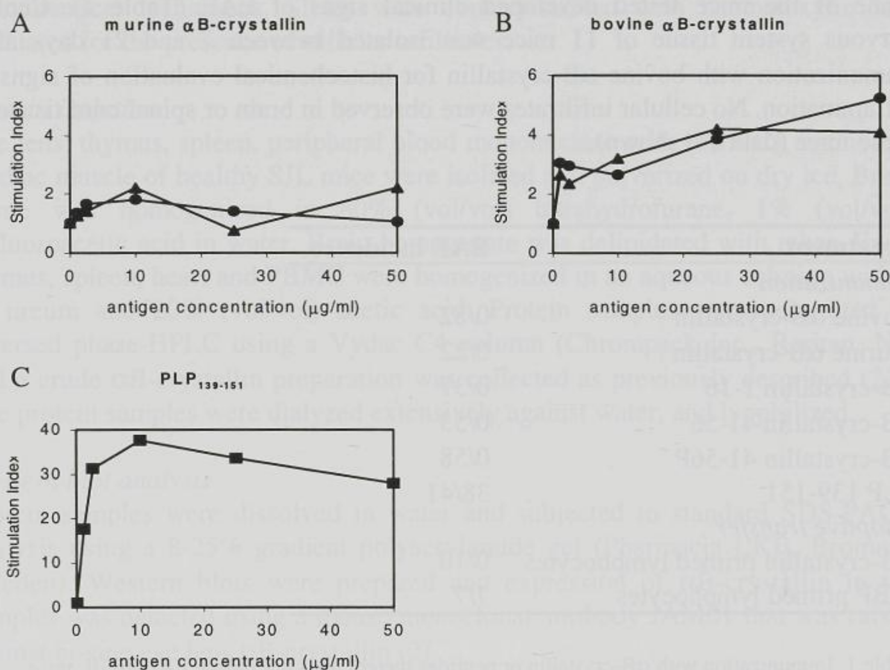

Figure 1. Proliferative responses of lymph node cells from mice that were immunized with (A) murine αB-crystallin, (B) bovine αB-crystallin, or (C) $PLP_{139-151}$. On day 0 and day 7, mice were immunized with 200 μg antigen in CFA. On day 21 after the first immunization, proliferative responses (stimulation indices) of lymph node cells to increasing concentrations of murine αB-crystallin (triangles), bovine αB-crystallin (circles), or $PLP_{139-151}$ (squares) were determined in a proliferation assay. Background counts per minute were 300, 158, and 864, respectively.

It is known from many models that EAE induction is often more successful when peptides instead of whole protein are used. Therefore, we also tested whether synthetic peptides corresponding to the previously identified T cell epitopes 1-16 and 41-56 of αB-crystallin (24) were encephalitogenic in SJL mice. Since T cells from SJL mice can discriminate between differentially phosphorylated forms of αB-crystallin (24), we also tested encephalitogenicity of 41-56P, a peptide that contains a phosphoserine at the natural phosphorylation site Ser_{45} (10). None of the protocols tested resulted in clinical signs of EAE, whereas almost all control animals that were immunized with $PLP_{139-151}$ developed clinically overt EAE (Table 1). Histochemical evaluation of central nervous system tissue of 4 to 6 mice out of each αB-crystallin peptide-immunized group revealed no signs of inflammation (data not shown). Also, adoptive transfer of 20-40 x 10^6 activated αB-crystallin-primed lymphocytes did not result in EAE, whereas transfer of similar numbers of activated MBP-specific lymphocytes induced severe EAE in all animals (Table 1).

To exclude that lack of encephalitogenicity of αB-crystallin was due to the absence of αB-crystallin as a target antigen in central nervous system myelin of mice, we isolated myelin proteins from brains of healthy SJL mice as previously described (2). Expression of αB-crystallin in mouse myelin was readily detectable by Western blotting (Figure 2).

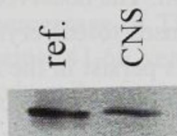

Figure 2. αB-Crystallin is present in central nervous system myelin from SJL mice. Myelin proteins were isolated from brains of healthy SJL mice and fractionated by reversed phase-HPLC. Western blot analysis was performed using an αB-crystallin-specific monoclonal antibody. Bovine αB-crystallin was used as a positive control.

αB-Crystallin is expressed in lymphoid tissue of SJL mice

Next, we analyzed αB-crystallin expression in primary and secondary lymphoid tissues that are involved in controlling immune reactivity to self proteins. Eye lens and cardiac muscle, tissues that constitutively express αB-crystallin, were used as positive controls. By reversed HPLC, a crude αB-crystallin protein preparation

was prepared from total protein extracts from thymus, spleen and peripheral blood mononuclear cells from healthy, unmanipulated mice. The presence of αB-crystallin in these tissue extracts was analyzed by Western blotting using an αB-crystallin specific monoclonal antibody (2). As shown in Figure 3, αB-crystallin could be detected in all tissues sampled.

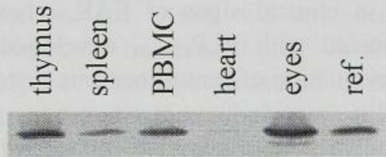

Figure 3. αB-Crystallin is expressed in lymphoid organs of SJL mice. Proteins were isolated from eye lens, thymus, spleen, PBMC, and cardiac muscle from healthy SJL mice. αB-Crystallin expression was assessed by Western blot analysis using an αB-crystallin-specific monoclonal antibody. Bovine αB-crystallin was used as a positive control.

SJL mice are functionally tolerant to αB-crystallin

The presence of αB-crystallin in lymphoid organs prompted us to study whether SJL mice are tolerant to this protein. The observed minor proliferative responses to the protein and the distinct responses to αB-crystallin peptides (24) indicate that some αB-crystallin reactive T cells persist in the SJL mouse T cell repertoire. Yet, functional tolerance to αB-crystallin may be accomplished by other mechanisms. We analyzed the cytokine profiles of T cell lines that are specific for the major T cell determinant 41-56 of αB-crystallin. This T cell determinant contains a natural phosphorylation site, Ser_{45} (25-27), and we have previously shown that T cells from SJL mice can discriminate between the phosphorylated and the unphosphorylated αB-crystallin determinant 41-56 (24). We therefore analyzed the T cell response to both determinants separately. SJL mice were immunized with either the unphosphorylated peptide 41-56 or the phosphorylated peptide 41-56P and lymph node cells from these mice were used to generate panels of T cells lines. Repeated restimulation in vitro with the respective peptides used for immunization resulted in 36 cell lines specific for 41-56 (cell lines O), and 39 cell lines specific for 41-56P (cell lines P). Production of IFN-γ and IL-4, as typical representatives of Th1 and Th2 cytokines, respectively, was measured in response to stimulation with the appropriate peptide. As can be seen in Figure 4, the vast

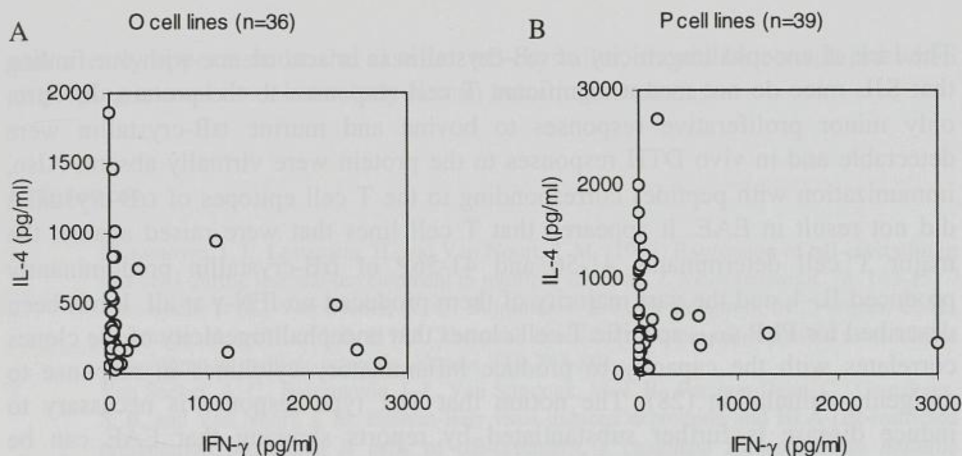

Figure 4. Cytokine profiles of αB-crystallin specific T cell lines. T cell lines were raised against the major T cell determinants αB-crystallin 41-56 (panel A, n=36) or 41-56P (panel B, n=39). Concentrations of IL-4 and IFN-γ (in pg/ml) in culture supernatants after stimulation with the respective peptides were determined by ELISA.

majority of T cell lines, i.e. over 85%, were of a Th2 type in that they produced high levels of IL-4 and minor amounts of IFN-γ, if any. Only a few T cell lines produced IFN-γ and no IL-4, and some produced IFN-γ as well as IL-4. There was no significant difference in the percentage of Th1 or Th2 type cell lines between the group of cell lines raised against 41-56 versus the group of cell lines raised against 41-56P. Control lymph node cells of mice that were primed with $PLP_{139-151}$ produced large amounts of IFN-γ as well as IL-4 in response to $PLP_{139-151}$ (data not shown).

Discussion

EAE can be induced with many different myelin proteins in rodents and is typically mediated by Th1 lymphocytes (11). The present study was aimed at investigating the encephalitogenic potential of the myelin-associated small heat shock protein αB-crystallin in mice. Despite extensive efforts using various different protocols that have proven to be successful in inducing EAE with other myelin proteins, no clinical or histological signs of EAE were observed after immunization with αB-crystallin or peptides representing its T-cell epitopes in 6 different strains of mice.

The lack of encephalitogenicity of αB-crystallin is in accordance with our finding that SJL mice do not mount significant T cell responses to the protein. In vitro, only minor proliferative responses to bovine and murine αB-crystallin were detectable and in vivo DTH responses to the protein were virtually absent. Also, immunization with peptides corresponding to the T cell epitopes of αB-crystallin did not result in EAE. It appeared that T cell lines that were raised against the major T cell determinants 41-56 and 41-56P of αB-crystallin predominantly produced IL-4, and the vast majority of them produced no IFN-γ at all. It has been described for $PLP_{139-151}$ specific T cell clones that encephalitogenicity of the clones correlates with the capacity to produce inflammatory cytokines in response to antigenic stimulation (28). The notion that Th1 type response is necessary to induce disease is further substantiated by reports showing that EAE can be ameliorated by inducing a shift from Th1 to Th2 cytokines (29, 30). The lack of encephalitogenic potential of αB-crystallin may thus be related to the inability of most T cell lines to produce IFN-γ in response to major T cell determinants of αB-crystallin.

The available data indicate that immunization of female SJL mice generally leads to a Th1 type T cell response (16, 17). Surprisingly, the vast majority of T cell lines that were raised against a T cell determinant of αB-crystallin did not produce IFN-γ, but rather produced IL-4. Control T cells, that were raised against $PLP_{139-151}$, produced large amounts of IFN-γ and IL-4. The lack of IFN-γ production in response to antigenic stimulation appeared to be a particular feature of αB-crystallin specific T cells from SJL mice and it corresponds with the presence of αB-crystallin in healthy lymphoid organs. Lymphoid expression generally leads to tolerance induction for a self protein. The fact that we were able to generate T cell lines specific for a major determinant of αB-crystallin indicates that not all αB-crystallin reactive T cells have been deleted or anergized. The risk of cellular autoimmunity in case αB-crystallin becomes upregulated under conditions of stress appears to be reduced by a naturally polarized Th2 type of response to αB-crystallin sequences. This mechanism of functional T cell tolerance has been termed immune deviation (31) and our findings suggest that it is functional in vivo to control autoreactivity against αB-crystallin.

In this report we have shown that SJL mice are functionally tolerant to αB-crystallin. It should be emphasized that human T cell lines are not functionally tolerant to αB-crystallin but instead respond to the protein by producing large amounts of IFN-γ and no IL-4 (2). Also in contrast to SJL mice, humans do not express αB-crystallin in healthy lymphoid tissue (3). Potential differences between humans and mice in expression and immune reactivity to self antigens, such as

now clearly appears to be the case for αB-crystallin, should be considered when employing animal models to study human autoimmune diseases.

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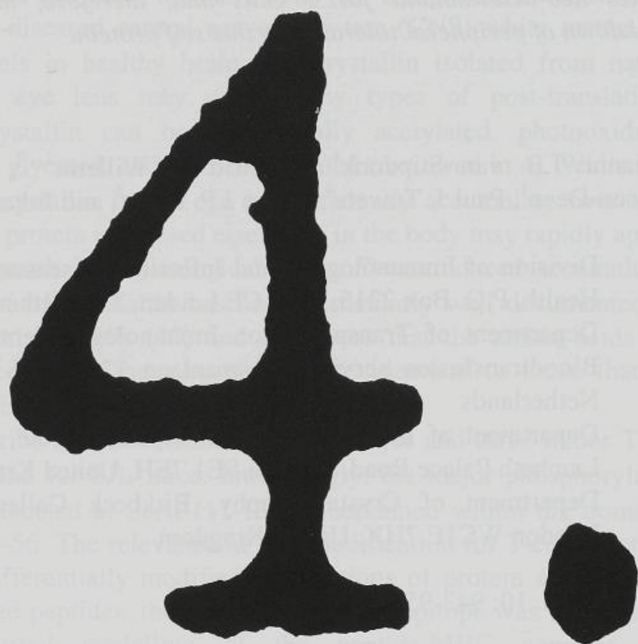
T cells discriminate between differentially phosphorylated forms of αB-crystallin, a major central nervous system myelin antigen



Summary

Factors such as developmental stage or physiological and infectious stress may change patterns of post-translational protein modification. In order to determine whether such regulated types of modification may influence T-cell responsiveness to self proteins we examined the T-cell response of SJL (H-2^s) mice to αB-crystallin, a small heat shock protein that can exist in differentially phosphorylated forms. Epitope mapping revealed the presence of two T-cell epitopes that are presented by I-A^s. One major epitope including residues 41-56 contains an amino acid residue (Ser$_{45}$) that can be phosphorylated as the result of aging or stress. Accordingly, T cells from SJL mice discriminate between preparations of αB-crystallin that differ in their extent of phosphorylation at the level of whole protein as well as at the level of determinant-specific responses. Phosphorylation at Ser$_{45}$ does not prevent binding of the peptide 41-56 to I-A^s and computer-assisted modelling of the peptide-MHC complex suggests that the phosphate group of the bound peptide extends outwards from the peptide-binding cleft and may thus be available for direct contact with T-cell receptors. Together, our data provide evidence that stress-inducible phosphorylation of αB-crystallin creates neo-determinants for T cells and, therefore, may contribute to the breakdown of peripheral tolerance to this self protein.

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Introduction

The elucidation of the crystal structures of an MHC class II molecule with peptide (1) and of the T cell receptor (2) have illustrated many details of how T cells recognize their antigenic target. A peptide interacts with the MHC molecule through the peptide backbone as well as via the amino acid side chains of the peptide that fit into haplotype-specific binding pockets of the MHC molecule. However, little is documented about the effect of naturally occurring post-translational modifications of protein antigens on their recognition by T cells. These modifications may affect antigen processing, MHC binding or interactions with the T cell receptor. The possibility that post-translational modifications may be specifically recognized by T cells may well have implications for the maintenance of tolerance to self antigens. The nature of post-translational modification of proteins may be influenced by several factors including age, physiological stress, disease, or cytokines (3-7). Consequently, under these conditions, neo-epitopes in self antigens may be generated for which the immune system may not be tolerant.

In 1995, it was shown that αB-crystallin, a small heat shock protein (8), is an immunodominant myelin antigen to human T cells (9). The protein is expressed at considerable levels in diseased central nervous system (CNS) white matter, but only at very low levels in healthy brain. αB-Crystallin isolated from natural sources such as the eye lens may carry many types of post-translational modifications. αB-Crystallin can be N-terminally acetylated, photooxidated, deamidated, truncated, glycated, and O-GlcNAcylated (reviewed in 4). While such modifications of αB-crystallin in the eye lens gradually accumulate over time, phosphorylation of the protein expressed elsewhere in the body may rapidly appear and disappear as the result of physiological stress. Stress-induced accumulation and phosphorylation of αB-crystallin has been particularly well documented for central nervous system glia cells (10) and it appears that the amino acids that become phosphorylated under these conditions are identical to those that are modified in eye lens αB-crystallin.

In this report we describe the identification of two major and three minor T cell epitopes of αB-crystallin for SJL mice. Interestingly, the major phosphorylation site of αB-crystallin, located at Ser_{45} (11-13), is contained within the dominant stimulatory peptide 41-56. The relevance of this modification for T-cell reactivity was assessed using differentially modified preparations of protein and peptides. Using a series of nested peptides, the core region of the epitope was identified as 43-52. Computer-assisted modelling of the peptide-MHC complex was subsequently applied to examine which of the amino acids within the core region

is likely to interact with T cell receptors. Evidence is presented that phosphorylation of αB-crystallin at Ser_{45} alters the protein at a position that is directly screened by I-A^s-restricted T cells.

Materials and Methods

Mice
Female SJL (H-2^s) mice were obtained from the Erasmus University, Rotterdam, The Netherlands and were used between 8 and 12 weeks of age.

Antigens
αB-Crystallin was purified from adult (viz. over one year of age) bovine eye lenses using reversed-phase HPLC as described previously (9). Briefly, bovine eye lenses were homogenized in 8 M urea, 20% (vol/vol) acetic acid in water and insoluble matter was removed by centrifugation. αB-Crystallin was purified to homogeneity by reversed-phase high-performance liquid chromatography using a ProRPC 15 μm column (Pharmacia Biotech, Uppsala, Sweden). The same purification protocol was applied to isolate the αB-crystallin subunit from a commercially available preparation of α-crystallin that is obtained from calves less than one year of age (Sigma Chemical Co., St. Louis, IL). In this study αB-crystallin purified from adult cattle is designated αB-$crystallin^A$ while the protein preparation obtained from young calves is referred to as αB-$crystallin^Y$.
Partially overlapping 16-mer peptides spanning the complete murine αB-crystallin sequence as well as the peptide containing phosphorylated Ser were made on an Abimed 422 robotic multiple synthesizer as peptide amides ($CONH_2$) as previously described (14).
Murine αB-crystallin differs from bovine αB-crystallin in 6 out of 175 positions. The sequences of the two major epitopes are identical between bovine and murine αB-crystallin. Within the three minor epitopes for SJL mice, substitutions (bovine→mouse) are present at positions 80 (Asn→Asp) and 152 (Ala→Val).

Immunizations
Mice were immunized s.c. at 2 sites in the flanks with an emulsion of 200 μg αB-$crystallin^A$ in CFA containing 1 mg/ml Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). In the case of peptide immunizations, mice were immunized s.c. at day 0 and at day 7 with an emulsion of 200 μg αB-crystallin peptide in CFA containing 5 mg/ml Mycobacterium tuberculosis H37RA.

Lymphocyte proliferation assay
Lymph node cells (LNC) were cyclically restimulated for 3 to 4 days, in the presence of irradiated (30 Gy) syngeneic splenocytes and thymocytes as antigen presenting cells (APC's), with 25 μg/ml αB-crystallin in culture medium (RPMI1640, Dutch modification; supplemented with 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 x 10^{-5} M β-mercaptoethanol with 5% (vol/vol) pooled human serum, and then propagated for 6 to 7 days in culture medium supplemented with 10% (vol/vol) FCS, IL-2 and 1% (vol/vol) non essential amino acids (Flow Laboratories, Irvine, UK).
Proliferative responses of LNC were measured in flat-bottom microtiter plates in triplicate cultures in a final volume of 0.2 ml culture medium. LNC were seeded at 5 x 10^4 per well in the presence of 2 x 10^5 APC's, 2% (vol/vol) mouse serum, and varying doses of antigen. Freshly isolated LNC were seeded at 2 x 10^5 cells per well in the presence of 10% (vol/vol) FCS, and varying doses of antigen. After 72 h of culture, 20 kBq [^{3}H]-thymidine was added to the cultures and after another 18 h, [^{3}H]-thymidine incorporation was determined using a ß-plate counter (Canberra Packard, Meriden, CT). Proliferative responses are expressed as stimulation indices.

Mass spectrometry
Lyophilized αB-crystallin^Y or αB-crystallin^A was redissolved in water/ methanol/acetic acid = 50/50/1 (vol/vol/vol) to a final concentration of 3 nmol/ml. Electrospray ionization mass spectrometry was performed on a hybrid quadrupole-time-of-flight mass spectrometer, the Q-TOF (Micromass, Manchester, UK). Samples were introduced by flow injection analysis using an on-line nanoflow electrospray interface (capillary spray tip of 20 μm inner diameter and 90 μm outer diameter) with an approximate flow rate of 300 nl/min. Injections of 1 μl were carried out by a FAMOS autosampler (LC Packings, Amsterdam, The Netherlands). Protein masses were calculated using the software supplied by the manufacturer together with the Q-TOF spectrometer.

Computer modelling
The model of the I-A^s molecule was built from that of the HLA-DR1 molecule (1, 15), using the COMPOSER suite of programs (16, 17) contained within SYBYL (Tripos Associates, St Louis, IL). In brief, the sequence of the I-A^s chains was built into the coordinates of the MHC class II α and β chains of HLA-DR1:influenza HA structure. Insertions and deletions relative to the DR1 sequence were built initially using the loop searching algorithms within COMPOSER and subjected to energy minimization. To model the peptide,

candidate residues for the P4 and P7 positions were used to align the test sequence within the binding cleft. Alignments that gave unacceptable contacts were eliminated and the remaining alignments were ranked by computer-assisted optimization of the remaining peptide side chain interactions with the MHC molecule, including the P1, P6 and P9 pockets. The optimal model was refined using a gradient torsional optimization algorithm. Initially the main chain of the molecule and the Cβ carbons were fixed. Following the convergence of the side refinement all of the atoms of the molecule were released and the optimization was performed on the entire model.

Results

Identification of T-cell epitopes of αB-crystallin for SJL mice

To identify epitopes of αB-crystallin, we measured the responsiveness of lymphocytes primed with αB-crystallin or peptides thereof to partially overlapping 16-mer peptides spanning the entire murine αB-crystallin sequence. SJL mice were immunized with αB-crystallin^{A} and following repeated stimulation of LNC in vitro, their peptide specificity was determined in a proliferation assay. As is shown in Figure 1, significant responses of αB-crystallin-specific LNC were recorded to peptides 1-16 and 41-56. In a parallel experiment, mice were immunized with pools of three partially overlapping peptides and draining LNC were tested for proliferative responses to each of the individual peptides. αB-Crystallin peptide primed LNC also responded strongly to αB-crystallin peptides 1-16 and 41-56 and to a lesser extent to peptides 73-88, 113-128 and 137-152 (data not shown).

We tested whether, perhaps in analogy to other findings (18), N-terminal acetylation of the αB-crystallin epitope 1-16 affects T cell reactivity towards this peptide. It appeared that T cells primed with the non-acetylated peptide fully crossreacted with the acetylated sequence and vice versa (data not shown). T cell proliferation assays with the use of a set of truncated and nested peptides revealed that the core region of this N-terminal epitope is located within residues 5-13 (data not shown). Computer modelling of peptide 1-16 in I-A^{s} confirmed that the N-terminus of the peptide does not play a role in interactions either with the MHC molecule or with the TcR (data not shown).

To determine the core region of the second major epitope contained in 41-56, SJL mice were immunized with peptide 41-56 and T-cell proliferative responses were tested against a set of partially overlapping peptides as represented in Figure 2.

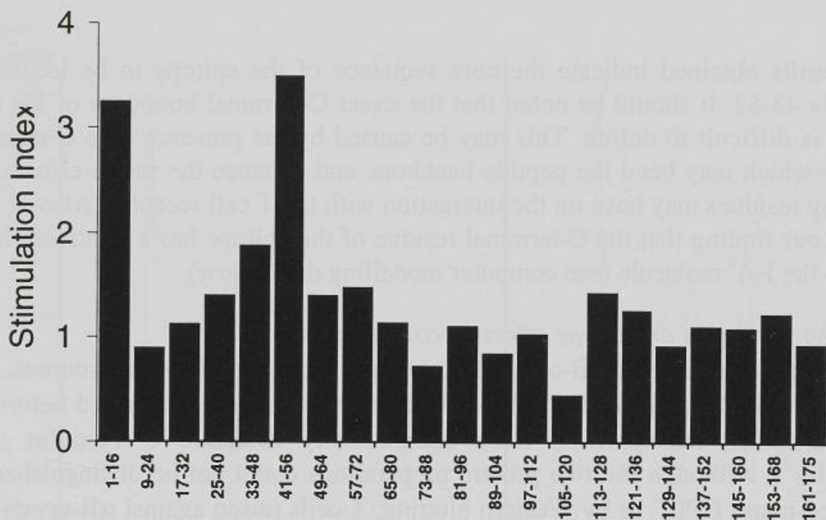

Figure 1. T cell epitopes of αB-crystallin for SJL mice. Proliferative responses of LNC to 25 μg/ml αB-crystallin peptides corresponding to the murine αB-crystallin sequence were determined in a proliferation assay. Mice were immunized with αB-crystallin/CFA and LNC were cyclically restimulated in vitro with 25 μg/ml αB-crystallin. The proliferative response to 25 μg/ml bovine αB-crystallin was 16.8 (s.i.).

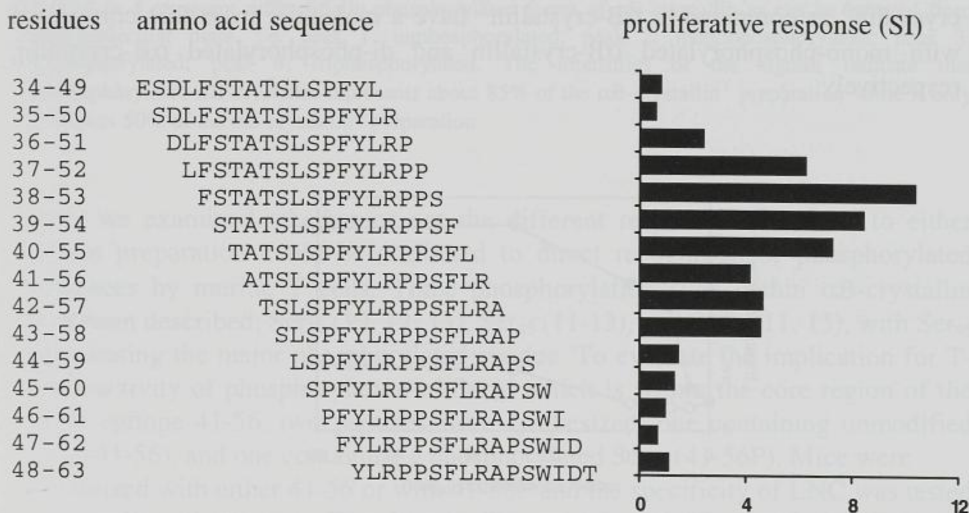

Figure 2. Delineation of the core region of T-cell epitope 41-56. Mice were immunized with 41-56/CFA and proliferative responses of LNC to 25 μg/ml of overlapping peptides were determined in a proliferation assay.

The results obtained indicate the core sequence of the epitope to be located at residues 43-52. It should be noted that the exact C-terminal boundary of the core region is difficult to define. This may be caused by the presence of a C-terminal proline which may bend the peptide backbone and enhance the subtle effects that flanking residues may have on the interaction with the T cell receptor. Also, it may reflect our finding that the C-terminal residue of the epitope has a relatively loose fit into the I-A^s molecule (see computer modelling data below).

Phosphorylation of an epitope affects recognition by T cells

In our studies, we used αB-crystallin obtained from two different sources; one (αB-crystallin^A) was isolated from adult bovine eye lenses as described before (9) and the other was purified from commercially available α-crystallin (αB-crystallin^Y). Although the two protein preparations could not be distinguished by reversed-phase HPLC or by Western blotting, T cells raised against αB-crystallin^A showed only a very limited cross-reactivity to the αB-crystallin^Y preparation (Figure 3). To examine whether this difference could be related to the state of phosphorylation of either preparation of αB-crystallin, both samples were submitted to mass spectrometry. This analysis shows that αB-crystallin^Y (Figure 4A) is more homogeneous than the αB-crystallin^A preparation (Figure 4B). The two additional protein variants (peaks 2 and 3) that are abundantly present in αB-crystallin^A as compared to αB-crystallin^Y have a molecular mass that correspond with mono-phosphorylated αB-crystallin and di-phosphorylated αB-crystallin, respectively.

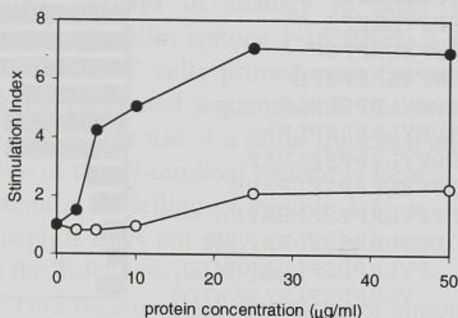

Figure 3. T cell responses to αB-crystallin^Y and αB-crystallin^A. Mice were immunized with αB-crystallin^A/CFA. Proliferative responses of LNC to increasing concentrations of αB-crystallin^Y (open circles) and αB-crystallin^A (closed circles) were determined in a proliferation assay.

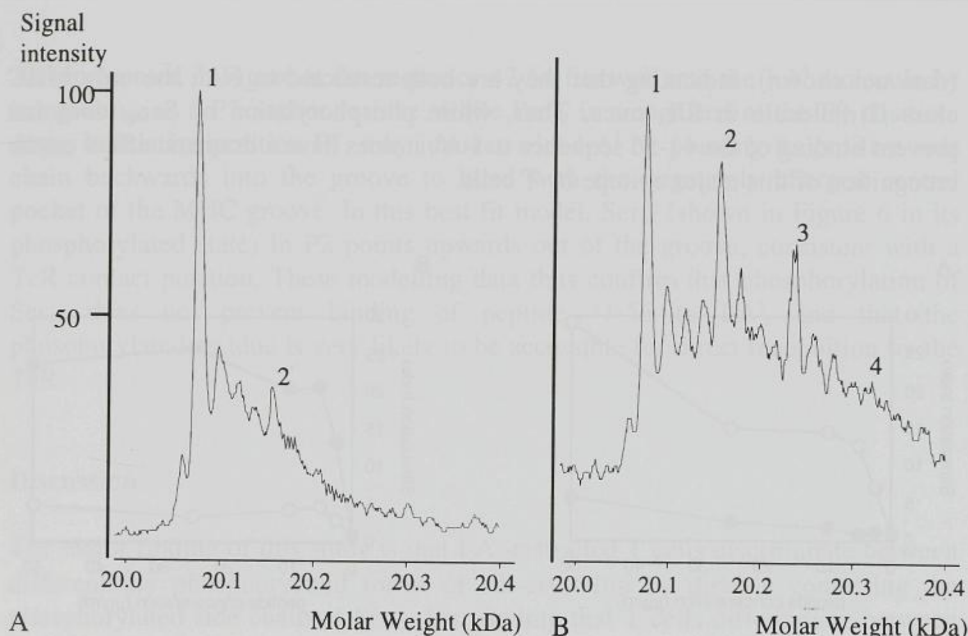

Figure 4. Analysis by mass spectrometry of αB-crystallin^{Y} (panel A) and αB-crystallin^{A} (panel B). Peaks 1 to 4 represent differentially phosphorylated forms of αB-crystallin as can be inferred from their molecular mass, i.e. peak 1: unphosphorylated; peak 2: monophosphorylated; peak 3: diphosphorylated; peak 4: triphosphorylated. The intensities of the signals indicate that unphosphorylated αB-crystallin represents about 85% of the αB-crystallin^{Y} preparation while it only represents 50% of the αB-crystallin^{A} preparation.

Next, we examined whether or not the different responses of LNC's to either protein preparation could be attributed to direct recognition of phosphorylated sequences by murine T cells. Three phosphorylation sites within αB-crystallin have been described; Ser_{19} (19, 12, 13), Ser_{45} (11-13), and Ser_{59} (11, 13), with Ser_{45} representing the major phosphorylated residue. To evaluate the implication for T-cell reactivity of phosphorylation of Ser_{45}, which is within the core region of the major epitope 41-56, two peptides were synthesized: one containing unmodified Ser_{45} (41-56), and one containing a phosphorylated Ser_{45} (41-56P). Mice were immunized with either 41-56 or with 41-56P and the specificity of LNC was tested in a proliferation assay. The data in Figures 5A and 5B reveal that both the phosphorylated and the unphosphorylated peptide triggered strong proliferative responses which displayed only limited cross reactivity to the other form. Responding T cell populations were $CD4^{+}CD8^{-}$ as detected by FACS analysis

(data not shown), indicating that they are both restricted to I-A^s, the only MHC class II molecule in SJL mice. Thus, while phosphorylation of Ser_{45} does not prevent binding of the 41-56 sequence to I-A^s, it does have a dramatic effect on the recognition of this major epitope by T cells.

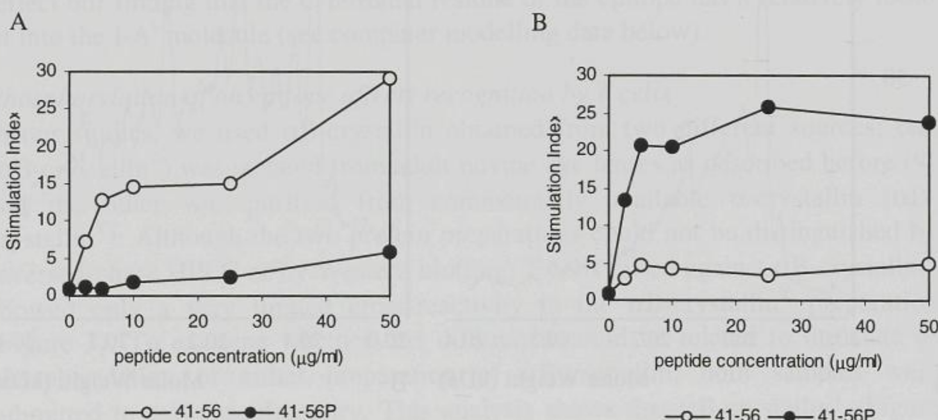

Figure 5. Phosphorylation of Ser_{45} affects T cell reactivity to αB-crystallin 41-56. Mice were immunized with either (A) 41-56/CFA or with (B) 41-56P/CFA. Proliferative responses of LNC to increasing concentrations of 41-56 (open circles) and 41-56P (closed circles) were determined in a proliferation assay.

Phosphorylated Ser_{45} is available for contacting T-cell receptors in the context of I-A^s

In order to examine whether murine T-cell receptors may directly contact the phosphorylated Ser_{45}, computer assisted modelling of the complex between the 42-54 epitope sequence and the I-A^s molecule was performed. Computer modelling of I-A^s indicates that the central region of its peptide binding cleft is dominated by two acid amino acid side chains of the β chain, viz. Asp_{28} and Glu_{72}. Consequently, binding of peptides containing basic residues at the appropriate positions P4 and/or P7 is favoured. Near the position of the C-terminus of bound peptides, the otherwise highly conserved residues Gln_{68} in the α chain and Trp_{61} in the β chain of MHC molecules are substituted in I-A^s by Thr and Tyr, respectively (20, 21). These substitutions abrogate hydrogen bonds to the peptide backbone that would otherwise be formed at these positions and thus, they will weaken the interaction between I-A^s and bound peptides at their C-termini.

As represented in Figure 6, the sequence 42-54 fits well into the I-A^s molecule by using Leu_{44} as a P1 anchor residue, while Pro_{52} is recognized at the P9 position. Arg_{50} in relative position P7, may interact with I-A^s by pointing its positive side chain backwards into the groove to bond with the negatively charged central pocket of the MHC groove. In this best fit model, Ser_{45} (shown in Figure 6 in its phosphorylated state) in P2 points upwards out of the groove, consistent with a TcR contact position. These modelling data thus confirm that phosphorylation of Ser_{45} does not prevent binding of peptide 41-56 to I-A^s, and that the phosphorylated residue is very likely to be accessible for direct recognition by the TcR.

Discussion

The major finding of this study is that I-A^s-restricted T cells discriminate between differentially phosphorylated forms of αB-crystallin by directly contacting the phosphorylated side chain of Ser_{45}. Our finding that T cells differentiate between phosphorylated and non-phosphorylated αB-crystallin does not appear to be a unique feature for I-A^s-restricted T cells since rat and human T cell lines can similarly discriminate between the two forms of αB-crystallin (M.J.B. van Stipdonk and A.C. van Sechel, manuscript in preparation). It is useful to note that murine T cells discriminate between the differentially phosphorylated sequences of αB-crystallin not only when given as a peptide, which may not need processing, but also when it is given as a protein. This indicates that the phosphate group is not removed upon passage through the endosomal processing compartment and indeed persists in the final MHC-presented form of the antigen.
Recently, it has been described that MHC class I restricted T cells may specifically recognize post-translationally altered forms of peptides (22, 23) and also carbohydrate specific T cells have been described (24). The types of modifications involved in these reports, however, are constitutive and unlikely to change over time. Thus, they will be part of the normal self antigenic repertoire. Stress-inducible modification on the other hand may have more severe consequences since it may generate neo-determinants in self proteins for which the immune system may not be tolerant. Indeed, it has recently been shown that proteins selectively phosphorylated during stress-induced apoptosis are prime targets of autoantibodies of patients with SLE (25).
In our studies, use was made of two αB-crystallin preparations that differed in their state of phosphorylation. While αB-$crystallin^Y$ contained only about 15% monophosphorylated protein, αB-$crystallin^A$ contained 50% of either mono- or

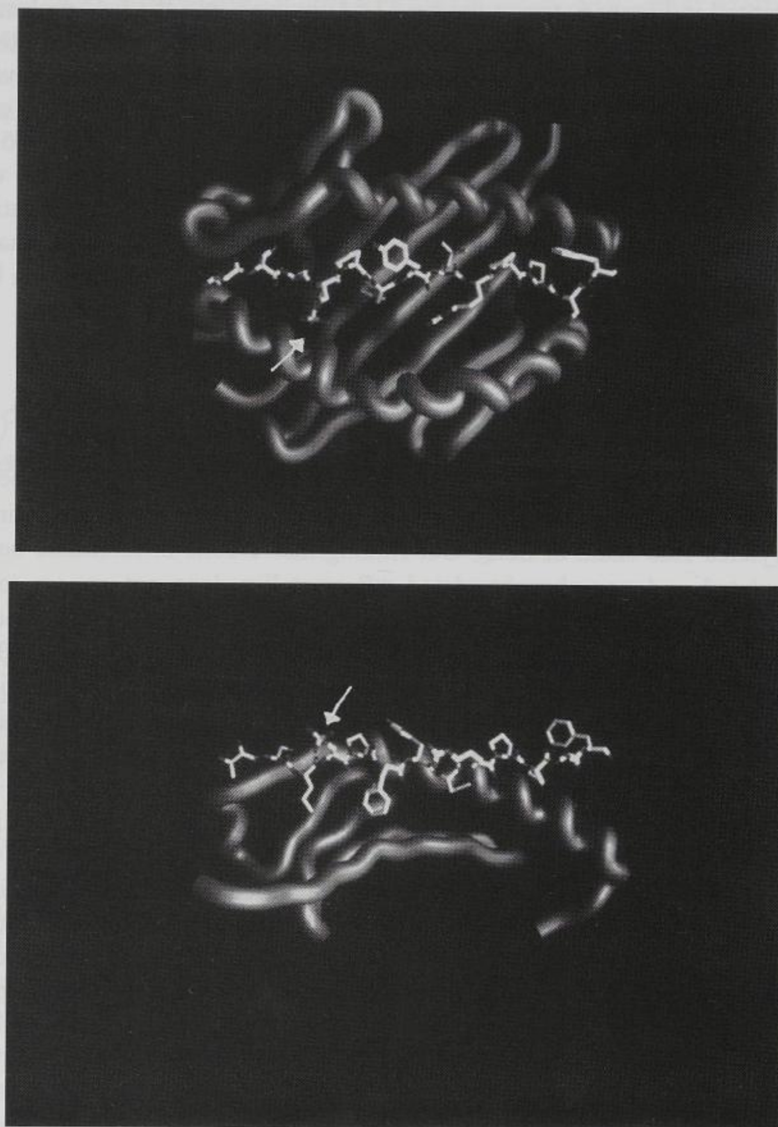

Figure 6. Model structure of the complex of I-A^s with αB-crystallin peptide 42-54P. Top (upper panel) and side (lower panel) views of the peptide : MHC complexes are shown, with the MHC molecule represented as a rope and the peptide shown in rod form to display the orientation of the side chains. The phosphorylated side chain of residue Ser_{45} (indicated by an arrow) can be seen to point upwards out of the peptide binding groove of the MHC molecule.

diphosphorylated protein. This is the result of differences in age of the animals used as a source (i.e. adult cattle for αB-crystallin^{A} versus calves less than one year old for αB-crystallin^{Y}). It is known that upon aging, lens crystallins accumulate a multitude of post-translational modifications, including phosphorylation (reviewed in 4). While the immune system may be relatively indifferent to modifications of αB-crystallin in the eye lens, an immune-privileged site, this may not apply to the same protein when it is expressed as a stress protein elsewhere in the body. As is the case for other small heat shock proteins (3, 26-29), phosphorylation of αB-crystallin is strongly increased under conditions of physiological stress (5-7). This stress-induced phosphorylation of αB-crystallin occurs at the same residues as in eye lens αB-crystallin (10). Rapid phosphorylation of pre-existing small heat shock proteins in response to stress precedes the intracellular accumulation of stress proteins resulting from increased protein synthesis (30). Yet, the physiological significance of sHSP phosphorylation remains to be established. Suggestions have been made that it could mediate protection against TNF-α induced cytotoxicity (3) but conflicting data have been reported on the effect of phosphorylation on the chaperone activity of αB-crystallin and cellular thermoresistance (7, 26, 31). Regardless of its physiological role, stress-induced phosphorylation of αB-crystallin results in the alteration of a determinant that can be specifically recognized by T cells as has been demonstrated in the present study. Taken together, our data point to the possibility that a stress-induced increase in expression and phosphorylation of αB-crystallin in an inflammatory environment may result in the generation of neo-epitopes for which the immune system may not be tolerant.

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5.

Partial T cell activation by a naturally phosphorylated determinant of αB-crystallin

Summary

αB-Crystallin is a small stress protein that is considered as a candidate autoantigen in multiple sclerosis. Under conditions of stress, expression of αB-crystallin is upregulated and the protein becomes highly phosphorylated. We have previously shown that in their proliferative response, murine T cells can discriminate between differentially phosphorylated forms of αB-crystallin. In this study we address qualitative aspects of this discrimination.
A panel of T cell lines were raised against either the unphosphorylated or the phosphorylated dominant T cell determinant 41-56 of αB-crystallin. These T cell lines were tested for reactivity to the two peptides at the level of both proliferation and cytokine secretion. Over 75% of the T cell lines showed limited or no cross-reactivity and proliferated predominantly in response to the peptide used for priming. Other cell lines were fully cross-reactive. In some cases, cell lines proliferated well in response to both peptides, but showed a dramatic difference in the production of IL-4 or IFN-γ in response to either peptide. Whereas the peptide used for priming induced cytokine production, the other peptide did not. Thus, a naturally occurring phosphorylation of a T cell determinant can lead to only partial T cell activation and thus, may qualitatively affect responses by T cells primed against the unphosphorylated determinant.

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Introduction

αB-Crystalline is highly expressed in central nervous system of multiple sclerosis patients (1) and it is an immunodominant myelin antigen to human T cells (2). Recently, it was shown that human αB-crystallin specific T cells can be activated in the periphery by Epstein Barr virus-transformed B cells, which express and present the protein (3). For these reasons αB-crystallin is thought to play a key role in the autoimmune pathology of multiple sclerosis (2, 4). αB-Crystallin is a small heat shock protein (5) and it is homologous to hsp 25, also a member of the family of small eukaryotic stress proteins (6). Like other heat shock proteins, αB-crystallin can function as a molecular chaperone (5, 7, 8), protecting the cell from harmful effects associated with various forms of physiological stress.
We have recently shown that murine T cells can discriminate between differentially phosphorylated forms of αB-crystallin (9). One of the natural phosphorylation sites of αB-crystallin, Ser_{45}, is located within a major T cell determinant for SJL ($H\text{-}2^s$) mice (9). Phosphorylation of αB-crystallin has been associated with stress-induced expression of the protein and is presumably mediated by MAPKAP kinase 2 and/or autophosphorylation (10-13). The physiological role of stress-induced phosphorylation of small heat shock proteins has not been established unequivocally (14-16). Stress-induced αB-crystallin purified from central nervous system glia cells can contain phosphorylated serines at position 19, 45 and 59 (10, 11). To the best of our knowledge, no information is available on the state of phosphorylation of αB-crystallin in various tissues of mice.
Phosphorylation of αB-crystallin does not interfere with binding of the determinant to the MHC molecule $I\text{-}A^s$, but rather, the phosphorylated residue can be directly contacted by T cell receptors (9). Several other groups have also reported that T cell receptors are able to recognize more than just bare peptide associated with a MHC molecule. T cells can for instance be specific for hapten-peptide (17), glycopeptide (18), or cysteinylated peptide (19). However, no studies have been reported on the question whether natural modification not only affects T cell reactivity in a quantitative but also in a qualitative way.
Individual T cell receptors may scan a peptide-MHC complex in a unique way and may differ in their dependence on particular side chains of the peptide for activation. When modification of an antigenic peptide occurs at a T cell receptor contact site, as is the case for phosphorylation of Ser_{45} of αB-crystallin, this may have important consequences for the ensuing T cell response. The effect of selective changes in a T cell determinant on the outcome of a T cell response has been extensively studied with the use of synthetic altered peptide ligands (20, 21).

Naturally altered peptide ligands may be derived from pathogens (22). Therapeutically, altered peptide ligands can be used to modulate the function of well-defined, e.g. autoreactive, T cell populations (23).
In the present study we addressed the question whether phosphorylation of a major T cell determinant of αB-crystallin can affect T cell responses in a qualitative manner. Our data indicate that at the level of individual T cell lines, this can indeed occur.

Materials and methods

Mice
Female SJL (H-2^s) mice were obtained from the Erasmus University, Rotterdam, The Netherlands and were used between 8 and 12 weeks of age.

Peptides
Synthetic peptides corresponding to the murine αB-crystallin sequence 41-56 (ATSLSPFYLRPPSFLR) were obtained from Genosys Biotechnologies (Cambridge, UK). The natural site of phosphorylation in this sequence is serine at amino acid position 45. The synthetic peptide containing a phosphate group at Ser_{45} is abbreviated as 41-56P.

Generation of T cell lines
At day 0 and at day 7 mice were immunized s.c. at 2 sites in the flanks with an emulsion of 200 μg αB-crystallin peptide in complete Freund's adjuvant containing 5 mg/ml Mycobacterium tuberculosis H37RA(Difco Laboratories, Detroit, MI). At day 21 after immunization, lymph node cells were isolated and restimulated in vitro with either 5 μg/ml peptide 41-56 (for cell lines O) or 5 μg/ml peptide 41-56P (for cell lines P) in culture medium (RPMI1640, Dutch modification; supplemented with 100 U ml/ml penicillin, 0.1 mg ml/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 x 10^{-5} M 2-ME) with 10% (vol/vol) FCS. After 4 days of culture, dead cells were removed using a Lympholyte-M (Cedar Lane, Hornby, Ontario, Canada) gradient. Viable cells were propagated in culture medium supplemented with 10% (vol/vol) FCS, IL-2 and 1% (vol/vol) non-essential amino acid solution (Flow Laboratories, Irvine, UK). After 7 days, lymphocytes were seeded at 2,000 cells/well or 20,000 cells/well in round-bottomed 96 well plates. These cell lines were cyclically restimulated for 3 to 4 days in the presence of 2 x 10^5 irradiated (30 Gy) syngeneic splenocytes and thymocytes as antigen presenting cells per well and peptide (see above) and then

propagated for 7 days in the presence of IL-2. Antigen specific cell lines (stimulation index greater than 2) were identified using a split well assay. Cell lines were cultured for 65 to 88 days before proliferation and cytokine production were analyzed.

Lymphocyte proliferation assay
Proliferative responses of cell lines were measured in round-bottomed microtiter plates in triplicate cultures in a final volume of 0.2 ml culture medium in the presence of 2 x 10^5 irradiated antigen presenting cells per well, 2% (vol/vol) mouse serum, and 5 µg/ml peptide 41-56 or peptide 41-56P. After 72 h of culture, 100 µl culture supernatant per well was removed for cytokine analysis and 20 kBq [^{3}H]-thymidine was added to the cultures. After another 18 h, [^{3}H]-thymidine incorporation was determined using a ß-plate counter (Canberra Packard, Meriden, CT). Proliferative responses are expressed as stimulation indices.

Cytokine ELISA
Culture supernatants were collected 72 hr after antigenic stimulation. The concentrations of IL-4 and IFN-γ in the supernatants were determined by standard ELISA technique using Quantigen™ sets of antibodies and recombinant cytokine standard (PharMingen, San Diego, CA) according to the manufacturer's protocol.

Results

We have shown previously that murine T cells can discriminate between differentially phosphorylated forms of the dominant T cell-determinant 41-56 of αB-crystallin. Proliferative responses of polyclonal T cell populations demonstrated no significant cross-reactivity between the phosphorylated and the unphosphorylated determinant (9). However, responses at the level of individual T cell lines were not examined. To address this issue we generated a panel of T cell lines and analyzed T cell proliferation and cytokine production in response to the unphosphorylated (peptide 41-56) and the phosphorylated determinant (peptide 41-56P). Responses to 5 µg/ml of either peptide were determined since in polyclonal lymphocyte proliferation assays this concentration resulted in nearly maximal response to the priming peptide and virtually no response to the other peptide (9). Limited cell numbers precluded analysis of additional peptide concentrations.
Cell lines were established by priming with either 41-56 (cell lines O) or 41-56P (cell lines P). Based on their cross-reactivity, cell lines were grouped into the following four categories: specific (exclusive proliferation to the priming peptide),

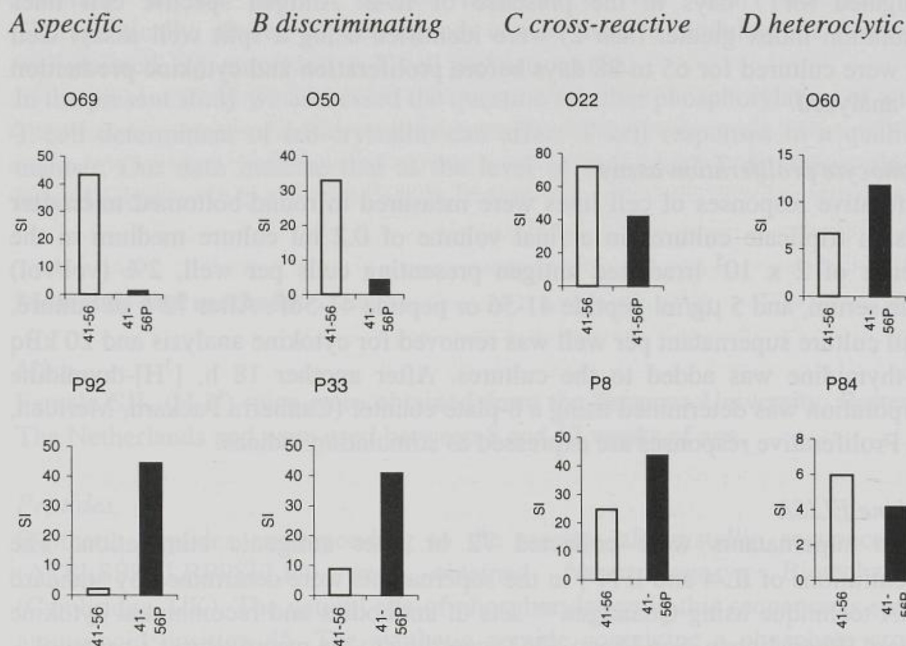


Figure 1. Division of αB-crystallin specific T cell lines into phosphorylation-state (A) specific, (B) discriminating, (C) cross-reactive, or (D) heteroclytic T cell lines. The proliferative response to 5 μg/ml 41-56 (white bars) or 5 μg/ml 41-56P (black bars) is given in stimulation indices. Upper panels are cell lines that were primed with 41-56, lower panels are cell lines that were primed with 41-56P. Background counts per minute were between 112 and 2232.

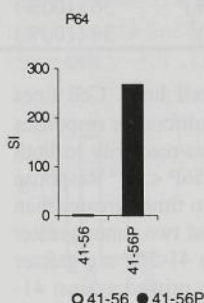
discriminating (predominant proliferation to the priming peptide), cross-reactive (comparable proliferation to both peptides), or heteroclytic (stronger responses to the peptide that was not used for priming). Representative examples of all four groups are given in Figure 1. Results of this analysis are summarized in Table 1.
Priming with either one of the two peptides led to the generation of many cell lines that were sensitive to the presence of absence of the phosphate group within the peptide. More than 75% of cell lines O and P responded at least two times stronger to the peptide used for priming than to the other peptide. At least 25% to 33% of the cell lines that were primed with either one of the two peptides did not proliferate at all in response to the other peptide. A small percentage (11% to 25%) of the T cell lines displayed cross-reactivity between the two peptides and some even responded stronger to the peptide different from the one used for priming.

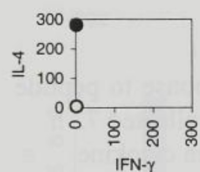
cell lines	priming peptide	specific	discriminating	cross-reactive	heteroclytic	total number
O	41-56	9 (25%)[1]	18 (50%)[3]	5 (14%)[5]	4 (11%)[6]	36 (100%)
P	41-56P	13 (33%)[2]	22 (56%)[4]	3 (8%)[5]	1 (3%)[7]	39 (100%)

Table 1. Phosphorylation-state dependence of αB-crystallin 41-56 specific T cell lines. Cell lines were raised against αB-crystallin 41-56 (cell lines O) or 41-56P (cell lines P). Proliferative responses to 41-56 and 41-56P were determined. Cell lines are grouped according to cross-reactivity to both peptides (stimulation indices). [1] Response to 41-56 > 41-56P and response to 41-56P < 2; [2] Response to 41-56P > 41-56 and response to 41-56 < 2; [3] Response to 41-56 is at least two times greater than response to 41-56P and response to 41-56P > 2; [4] Response to 41-56P is at least two times greater than response to 41-56 and response to 41-56 > 2; [5] Responses to 41-56 and to 41-56P are greater than 2 and differ less than a factor of 2; [6] Response to 41-56P > 41-56 for T cells primed against 41-56; [7] Response to 41-56 > 41-56P for T cells primed against 41-56P.

Next, we analyzed cytokine production by the T cell lines in response to peptide 41-56 and peptide 41-56P. Culture supernatants of cell lines were collected 72 h after stimulation with either one of the peptides, the time point when cytokine concentrations are optimal under our experimental conditions. Production was determined of IFN-γ and IL-4, as representatives of Th1 and Th2 responses, respectively. The vast majority of T cell lines displayed a Th2 type of response in that they produced IL-4, but no IFN-γ, regardless of whether they were primed with unphosphorylated or phosphorylated 41-56. For most cell lines, stronger proliferation in response to a peptide correlated with a higher concentration of cytokine in the corresponding culture supernatant (Figure 2). However, some cell lines proliferated well in response to both peptides but showed a dramatic difference in cytokine production in response to either of the two different peptides. Examples of cell lines in which the peptide used for priming induced cytokine production, whereas the variant peptide did not, are given in Figure 3. For example, cell line O22, that was primed with 41-56, proliferated well in response to both 41-56 and 41-56P (stimulation indices 72 and 42, respectively). But, whereas stimulation with peptide 41-56 resulted in the production of high levels of IFN-γ, virtually no IFN-γ was detectable after stimulation with 41-56P. Cell line P8 that was primed with 41-56P, an example of a Th2 cell line, also proliferated well in response to both peptides, but only produced IL-4 in response to 41-56P, but not in response to 41-56.

A specific





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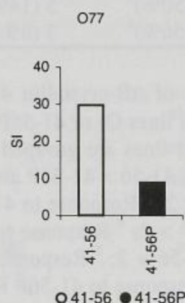

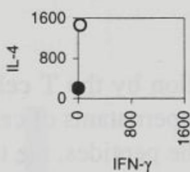

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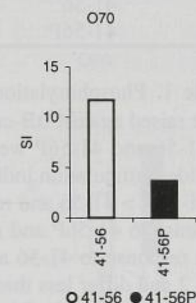
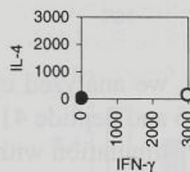
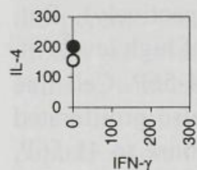
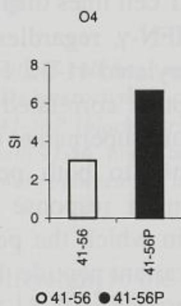




C cross-reactive



D heteroclytic



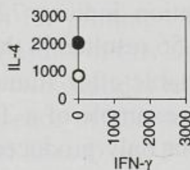


Figure 2. T cell lines for which cytokine production correlates with proliferative responses to peptides 41-56 and 41-56P. Representative examples of proliferation (upper panels) and cytokine production (lower panels) of phosphorylation-state (A) specific, (B) discriminating, (C) cross-reactive, or (D) heteroclytic T cell lines in response to 5 μg/ml 41-56 (white bars/dots) or 5 μg/ml 41-56P (black bars/dots). Proliferation is expressed as stimulation indices and cytokine production is expressed in pg/ml. Background counts per minute were between 64 and 3721.

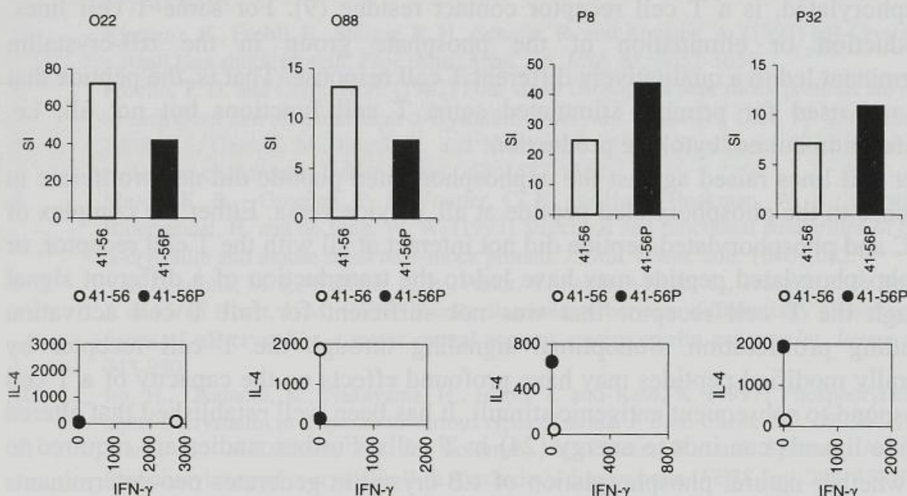


Figure 3. Cross-reactive T cell lines for which proliferation and cytokine production are uncoupled. Whereas proliferation of the T cell line is stimulated by both peptides, cytokine production is only induced by the peptide used for priming. Representative examples of proliferation (upper panels) and cytokine production (lower panels) of cross-reactive T cell lines in response to 5 μg/ml 41-56 (white bars/dots) or 5 μg/ml 41-56P (black bars/dots). Proliferation is expressed as stimulation indices and cytokine production is expressed in pg/ml. Background counts per minute were between 112 and 1544. Cell lines O were primed with 41-56 and cell lines P were primed with 41-56P.

Discussion

The major finding of this study is that a naturally occurring phosphorylation of a self determinant of αB-crystallin can stimulate T cells primed by the unphosphorylated determinant to proliferate but not to produce cytokines. In other words, natural alterations in self proteins may result in a qualitatively different T cell response.

The panel of T cell lines that were raised against the unphosphorylated and phosphorylated αB-crystallin determinants were heterogeneous in their dependence on the absence or presence of a phosphate group. Some cell lines proliferated exclusively in response to the priming peptide, whereas other cell lines were fully cross-reactive to the two peptides. Most T cell lines that were raised against the unphosphorylated determinant were not cross-reactive to the phosphorylated determinant, and vice versa. These findings are in agreement with computer modeling data that indicate that Ser_{45}, the amino acid that can be

phosphorylated, is a T cell receptor contact residue (9). For some T cell lines, introduction or elimination of the phosphate group in the αB-crystallin determinant led to a qualitatively different T cell response. That is, the peptide that was not used for priming stimulated some T cell functions but not all, i.e. proliferation but not cytokine production.
Other cell lines raised against the unphosphorylated peptide did not proliferate in response to the phosphorylated peptide at all, or vice versa. Either the complex of MHC and phosphorylated peptide did not interact at all with the T cell receptor, or the phosphorylated peptide may have led to the transduction of a different signal through the T cell receptor that was not sufficient for full T cell activation including proliferation. Suboptimal signaling through the T cell receptor by naturally modified peptides may have profound effects on the capacity of a T cell to respond to subsequent antigenic stimuli. It has been well established that altered peptide ligands can induce anergy (24) in T cells. Further studies are required to test whether natural phosphorylation of αB-crystallin generates neo-determinants that can lead to anergy induction in T cells.
Thus, a stress-induced increase in expression and phosphorylation of αB-crystallin may not only stimulate a set of T cells that are specific for phosphorylated αB-crystallin, but it may also affect responses of T cells that were primed by unphosphorylated αB-crystallin. Similar to what we have shown for murine T cells (9), human T cells can also discriminate between differentially phosphorylated forms of αB-crystallin (Van Sechel, personal communication). Therefore the phenomena described here may also be of relevance to human T cell responses to naturally phosphorylated forms of αB-crystallin.

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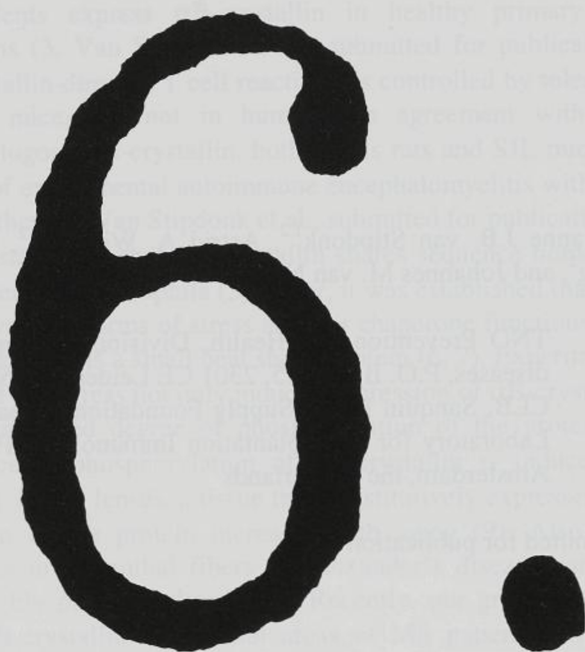
T cell tolerance for all differentially phosphorylated forms of αB-crystallin in rodents



Summary

αB-Crystallin is a small stress protein that is considered as a candidate autoantigen in multiple sclerosis. Under conditions of stress, αB-crystallin becomes phosphorylated. We have previously shown that murine T cells can discriminate between differentially phosphorylated forms of heterologous αB-crystallin. We now analyzed the phosphorylation state of αB-crystallin as it is expressed in various organs of rats and mice. Surprisingly, even in the apparent absence of stress, healthy rodent tissues contain phosphorylated forms of αB-crystallin. The capacity of the murine T cell repertoire to respond to unphosphorylated versus phosphorylated self αB-crystallin was investigated both in vivo and in vitro. We here show that T cell tolerance to autologous αB-crystallin applies to both unphosphorylated and phosphorylated forms of the protein.

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Introduction

Analysis of T cell reactivity to the complete collection of central nervous system (CNS) myelin proteins from multiple sclerosis (MS)-affected brains, demonstrated that human peripheral blood mononuclear cells (PBMC) respond dominantly and in a pro-inflammatory way to αB-crystallin (1). Subsequent immunohistochemical evaluation of CNS tissue of MS patients revealed the presence of elevated levels of αB-crystallin in MS-affected areas of the brain (1, 2). Recently, we have shown that viral infection of human B cells induces expression of αB-crystallin and HLA-DR-restricted presentation of αB-crystallin sequences to human T cells (3). Thus, autoreactive αB-crystallin specific T cells may be activated peripherally. For these reasons, αB-crystallin is considered as a candidate autoantigen in MS (reviewed by Van Noort et al., 4).

Rodents differ markedly from humans with respect to cellular reactivity to αB-crystallin. Both in vivo delayed type hypersensitivity (DTH) responses and in vitro proliferative responses to autologous αB-crystallin were virtually undetectable in Lewis rats and SJL mice (Van Stipdonk et al., submitted for publication). In contrast to humans, rodents express αB-crystallin in healthy primary and secondary lymphoid organs (3, Van Stipdonk et al., submitted for publication). Correspondingly, αB-crystallin-directed T cell reactivity is controlled by tolerance in Lewis rats and SJL mice, but not in humans. In agreement with this nonresponsiveness to autologous αB-crystallin, both Lewis rats and SJL mice are resistant to the induction of experimental autoimmune encephalomyelitis with αB-crystallin or determinants thereof (Van Stipdonk et al., submitted for publication).

Already in 1982, it was established that αB-crystallin shares sequence homology with small heat shock proteins of Drosophila (5). Later, it was established that αB-crystallin is inducible by various forms of stress and has chaperone functions, and therefore can indeed be regarded as a small heat shock protein (6, 7). Experimental evidence has demonstrated that stress not only induces expression of αB-crystallin, but it also leads to an enhanced degree of phosphorylation of the protein. In cultured human glioma cells, phosphorylation of αB-crystallin is induced by various forms of stress (8). In eye lenses, a tissue that constitutively expresses αB-crystallin, phosphorylation of the protein increases with aging (9). Also, αB-crystallin that accumulates in Rosenthal fibers of Alexander's disease patients' brain was found to be highly phosphorylated (10). Recently, our group detected highly phosphorylated αB-crystallin in lesional areas of MS patients, whereas unaffected CNS tissue almost exclusively contained unphosphorylated αB-crystallin (manuscript in preparation). Thus, both in vivo and in vitro,

phosphorylation is clearly associated with stress-induced expression of the protein, but the physiological relevance of phosphorylation of αB-crystallin has not been established yet. Serines at position 19, 45, and 59 have been identified as natural phosphorylation sites of bovine and human αB-crystallin (8, 9, 11, 12).
One of these phosphorylation sites, Ser_{45}, is contained within the major T cell determinant 41-56 of αB-crystallin for SJL mice. We have previously shown that murine T cells discriminate between differentially phosphorylated forms of αB-crystallin, both at the level of responses to bovine protein and at the level of responses to epitope 41-56 (13). As discussed above, we have also demonstrated that T cell reactivity to autologous αB-crystallin is controlled by tolerance (Van Stipdonk et al., submitted for publication). However, the grade of phosphorylation of the autologous αB-crystallin preparation that was used for these studies was not identified. This thus raises the question whether tolerance applies equally to all different phosphorylated forms of autologous αB-crystallin.
In the present study, we first analyzed the phosphorylation state of αB-crystallin in various healthy rodent tissues. It appeared that even in the apparent absence of stress, αB-crystallin is highly phosphorylated in several organs of mice and rats. Next, we analyzed T cell reactivity to either unphosphorylated or phosphorylated murine αB-crystallin both in vivo and in vitro. Data presented indicate that tolerance applies to all differentially phosphorylated forms of autologous αB-crystallin.

Materials and Methods

Animals

Male Lewis/CrIBr rats were obtained from Charles River/the Broekman Institute, Someren, the Netherlands. Female SJL mice were obtained from the Erasmus University, Rotterdam, The Netherlands. All animals were housed under specific pathogen free (spf) conditions in the animal facility of TNO Prevention and Health, Leiden, the Netherlands and had free access to acidified water (pH 2.2) and irradiated food pellets. All animals were used between 6 and 12 weeks of age.

Protein isolation

Eye lenses, thymus, spleen, peripheral blood mononuclear cells (PBMC), brain, and cardiac muscle of healthy, young adult mice and rats were isolated and pulverized on dry ice. Brain tissue was homogenized in 80% (vol/vol) tetrahydrofurane, 1% (vol/vol) trifluoroacetic acid in water. Brain homogenate was

delipidated with ether. Eyes, thymus, spleen, heart and PBMC were homogenized in an aqueous solution containing 8 M ureum and 20% (vol/vol) acetic acid. Protein samples were submitted to reversed phase-HPLC using a Vydac C4 column (Chrompack Inc., Raritan, NJ) and a crude αB-crystallin preparation was collected as previously described (14).
Differentially phosphorylated forms of αB-crystallin were separated using a MonoQ H5/5 anion exchange chromatography column (Pharmacia LKB, Bromma, Sweden) as recently described (14). The buffer used was 25 mM Tris-HCl, 8M ureum in water, pH 8.8 and proteins were eluted by increasing concentrations of NaCl. Fractions that eluted between 0 mM and 150 mM NaCl were collected, dialyzed extensively against water, and lyophilized.

Western blot analysis
Protein samples were dissolved in water and subjected to standard SDS-PAGE analysis using a 8-25% (weight/vol) gradient polyacrylamide gel (Pharmacia LKB, Bromma, Sweden) and blotted onto nitrocellulose filters. For detection of αB-crystallin in mouse tissue samples, mouse monoclonal antibody JAM01 that was raised against bovine αB-crystallin, was used (1). The presence of αB-crystallin in rat samples was detected using mouse monoclonal antibody Spa 223 (StressGen, Victoria, Canada) that was raised against the C-terminal peptide 160-175 of αB-crystallin. Both antibodies recognize αB-crystallin irrespective of its phosphorylation state.

Immunizations
On day 0 and day 7, SJL mice were immunized s.c. in the flanks with 200 μg αB-crystallin or αB-crystallin peptide emulsified in CFA (containing 5 mg/ml Mycobacterium tuberculosis, Difco Laboratories, Detroit, MI). Unphosphorylated and phosphorylated αB-crystallin was isolated from murine eye lenses as described above. Phosphorylated αB-crystallin contained a mixture of mono-, di, and tri-phosphorylated αB-crystallin as they are present in murine eye lenses. Synthetic peptides corresponding to the murine αB-crystallin sequence 41-56 (ATSLSPFYLRPPSFLR) were obtained from Genosys Biotechnologies (Cambridge, UK). The natural site of phosphorylation in this sequence is serine at amino acid position 45. The synthetic peptide containing a phosphate group at Ser_{45} is abbreviated as 41-56P.

Lymphocyte proliferation assay
Proliferative responses of freshly isolated lymph node cells were measured in flat-bottomed microtiter plates in triplicate cultures in a final volume of 0.2 ml culture

medium medium (RPMI1640, Dutch modification; supplemented with 100 U ml/ml penicillin, 0.1 mg ml/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 x 10^{-5} M 2-ME). Lymph node cells were seeded at 2 x 10^{5} cells/well in the presence of 10% (vol/vol) FCS, and varying doses of antigen. After 72 h of culture, 20 kBq [^{3}H]-thymidine was added to the cultures. After another 18 h, [^{3}H]-thymidine incorporation was determined using a ß-plate counter (Canberra Packard, Meriden, CT). Proliferative responses are expressed as stimulation indices.

Delayed Type Hypersensitivity (DTH) measurement

Mice were challenged with 100 µg protein or peptide (in 50 µl PBS) in the hind foot. The other hind foot was injected with 50 µl PBS alone and served as a control. DTH reactions were determined 24 h and 48 h after challenge, using a pressure-sensitive micrometer (Mitutoyo, Tokyo, Japan). Values are expressed as (mm $foot_{antigen}$ - mm $foot_{PBS}$).

Results

Highly phosphorylated αB-crystallin is present in healthy tissues of mice and rats

As a first step, we investigated the phosphorylation state of αB-crystallin in healthy rodent tissues. We took a particular interest in lymphoid organs because these play a key role in processes of immunological tolerance induction. Brain tissue was included in the study because many pathologies of the human central nervous system have been shown to lead to overexpression and abnormal modification of αB-crystallin. Eye lenses and cardiac muscle were analyzed because these organs are known to express high levels of αB-crystallin. The tissues were isolated from healthy, young adult (age 6 to 12 weeks) SJL mice and Lewis rats.

Analysis of the phosphorylation state of αB-crystallin in the tissue samples by isoelectric focussing was severely hampered by spontaneous multimerization of αB-crystallin that occurs in vitro (15, and our own data not shown). Thus both unphosphorylated and phosphorylated αB-crystallin aggregates in vitro into large multimers that preclude the analysis of monomers under native conditions. Therefore we separated differentially charged forms of αB-crystallin by ion exchange chromatography under strongly denaturing conditions, i.e. 8 M urea. Proteins eluting between 0 and 150 mM NaCl were collected in 18 separate fractions. The contents of these fractions were then analyzed by Western blotting

for the presence of αB-crystallin using αB-crystallin specific monoclonal antibodies. This method has been described previously for the isolation of differentially phosphorylated forms of αB-crystallin purified from bovine eye lenses (14). As determined by mass spectrometry, unphosphorylated αB-crystallin is collected in fractions 3 and 4, mono-phosphorylated αB-crystallin in fractions 7 and 8, di-phosphorylated αB-crystallin is present in fraction 10 and 11, and finally tri-phosphorylated αB-crystallin ends up in fraction 14.
Analysis of eye lenses of mice revealed the presence of different phosphorylated forms of αB-crystallin similar to what can be found in adult bovine eye lenses (Figure 1). The protein bands of lesser molecular weight than the main 20 kD band that are stained by the antibody correspond to truncated forms of αB-crystallin (16). In the thymus, brain and cardiac muscle unphosphorylated αB-crystallin predominates (Figure 1). Apart from unphosphorylated αB-crystallin, also considerable amounts of mono-phosphorylated αB-crystallin could be detected in spleen tissue (Figure 1). In PBMC all forms of αB-crystallin, including highly phosphorylated protein, were detectable (Figure 1). Thus, in different tissues of SJL mice considerable differences exist between the phosphorylation status of αB-crystallin and surprisingly, highly phosphorylated forms of αB-crystallin can be found in healthy tissues.
Also, eye lenses of Lewis rats were found to contain both unphosphorylated and phosphorylated forms of αB-crystallin (Figure 2). In cardiac muscle, the majority of αB-crystallin was recovered in fractions 3 and 4 (Figure 2). Small amounts of αB-crystallin were detectable in spleen and PBMC of Lewis rats. In spleen, αB-crystallin was predominantly unphosphorylated, whereas in PBMC highly phosphorylated forms of αB-crystallin were found (Figure 2). Both thymus and brain of Lewis rats contain high levels of unphosphorylated as well as highly phosphorylated αB-crystallin (Figure 2). Thus, phosphorylation of αB-crystallin differs markedly between different tissues of healthy Lewis rats, and again, highly phosphorylated forms of the protein can be found in the apparent absence of stress.
Patterns of phosphorylation of αB-crystallin in various organs are largely comparable between mice and rats. Highly phosphorylated αB-crystallin is present at considerable levels in brain and thymus of rats but not in mice. Spleen of mice clearly contains mono-phosphorylated αB-crystallin, whereas only low levels of predominantly unphosphorylated protein was detectable in rats. Highly phosphorylated αB-crystallin was found in PBMC of both rats and mice.

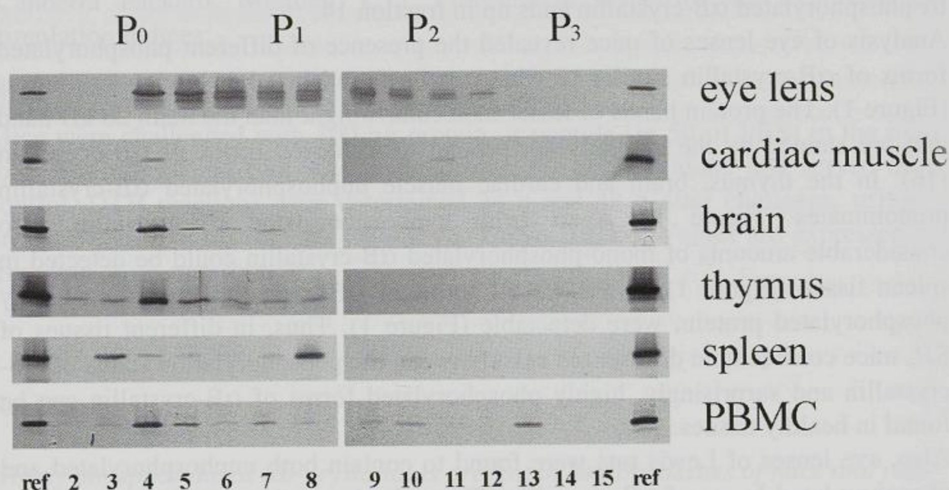

Figure 1. Distinct patterns of phosphorylation of αB-crystallin in various tissues of healthy, young adult SJL mice. Proteins were submitted to ion exchange chromatography and the resulting fractions were analyzed by Western blotting using monoclonal antibody JAM01 that was raised against bovine αB-crystallin. Tissues analyzed include eye lens, cardiac muscle, brain, thymus, spleen, and PBMC. αB-Crystallin purified from bovine eye lens is used as a reference. Fraction numbers and the locations of unphosphorylated (P_0), mono-phosphorylated (P_1), di-phosphorylated (P_2), and tri-phosphorylated (P_3) αB-crystallin in the elution profile are indicated.

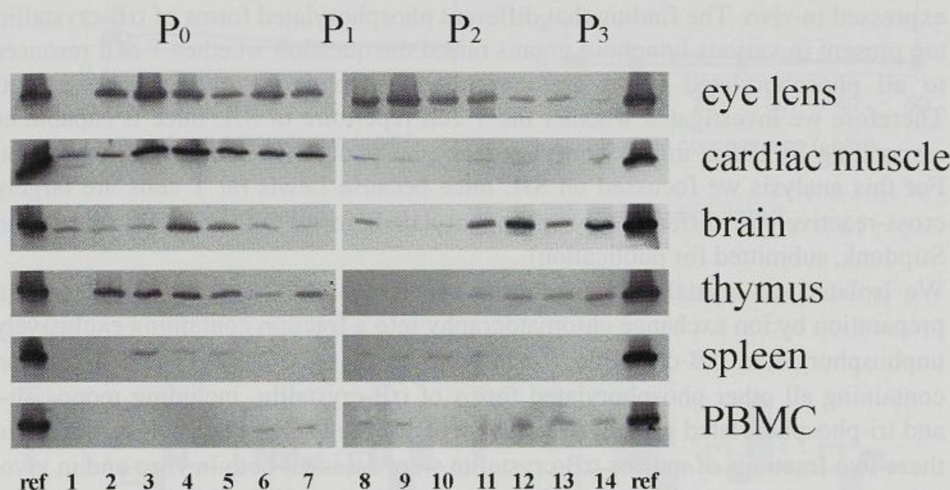

Figure 2. Distinct patterns of phosphorylation of αB-crystallin in various tissues of healthy, young adult Lewis rats. Proteins were submitted to ion exchange chromatography and the resulting fractions were analyzed by Western blotting using monoclonal antibody Spa223 that was raised against the C-terminal peptide 160-175 of αB-crystallin. Tissues analyzed include eye lens, cardiac muscle, brain, thymus, spleen, and PBMC. αB-Crystallin purified from bovine eye lens is used as a reference. Fraction numbers and the locations of unphosphorylated (P_0), mono-phosphorylated (P_1), di-phosphorylated (P_2), and tri-phosphorylated (P_3) αB-crystallin in the elution profile are indicated.

SJL mice are tolerant to both unphosphorylated and phosphorylated αB-crystallin
We have previously shown that T cells from SJL mice discriminate between differentially phosphorylated forms of αB-crystallin, both at the level of responses to whole bovine protein, as well as at the level of responses to a major T cell determinant of αB-crystallin 41-56 (13). Thus different T cell populations may respond to the different phosphorylated forms of αB-crystallin as they are expressed in vivo. The finding that different phosphorylated forms of αB-crystallin are present in various lymphoid organs raised the question whether T cell resonses to all phosphorylated forms are controlled by tolerance to the same extent. Therefore we investigated whether the T cell repertoire of SJL mice is capable of responding to either unphosphorylated or phosphorylated murine αB-crystallin. For this analysis we focussed on SJL mice because Lewis rat T cells are largely cross-reactive to differentially phosphorylated forms of αB-crystallin (Van Stipdonk, submitted for publication).
We isolated αB-crystallin from murine eye lenses and fractionated the protein preparation by ion exchange chromatography into a fraction containing exclusively unphosphorylated αB-crystallin (fractions 3 to 5, see Figure 1) and a fraction containing all other phosphorylated forms of αB-crystallin, including mono-, di-, and tri-phosphorylated protein (fractions 6 to 15, see Figure 1). T cell responses to these two fractions of murine αB-crystallin were assessed both in vitro and in vivo. Mice were immunized with either unphosphorylated or phosphorylated αB-crystallin and antigen-specific responses of draining lymph node cells were determined in a proliferation assay. Lymphocytes of both groups of mice showed no specific proliferative response to either form of αB-crystallin (Figure 3 A and B). As a control, responses were also determined to Mycobacterium tuberculosis, a component of the adjuvant used for immunization (Figure 3 A and B). We analyzed in vivo responses by evaluating delayed type hypersensitivity (DTH) responses. In accordance with the in vitro data, no significant DTH responses could me measured in response to either form of αB-crystallin (Figure 3 C and D).
Notwithstanding the lack of T cell reactivity to murine αB-crystallin following immunization with whole protein, we have previously shown T cell responses to occur to peptides derived from murine αB-crystallin (13). One of the major T cell determinants of αB-crystallin, viz. the sequence 41-56, contains a natural phosphorylation site that can be specifically recognized by I-A^s-restricted T cells from SJL mice. As another control, we therefore investigated the immune response in SJL mice to either the unphosphorylated (peptide 41-56) or the phosphorylated (peptide 41-56P) T cell determinant of αB-crystallin. Lymph node cells of peptide-immunized mice proliferated strongly in response to the peptide that was used for

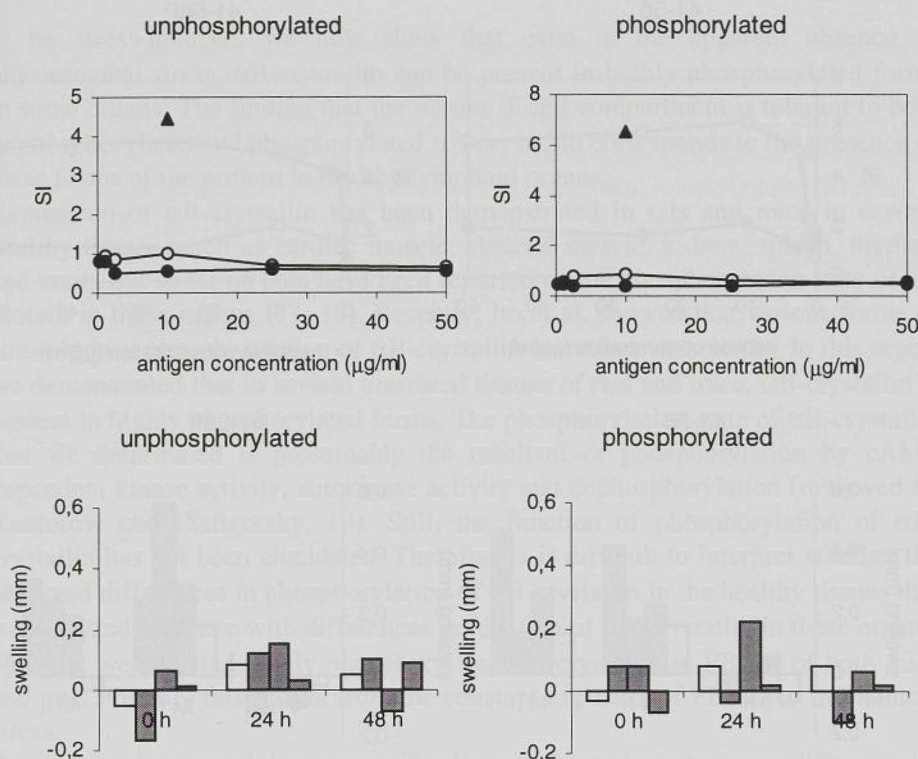

Figure 3. Absence of T cell reactivity to either unphosphorylated or phosphorylated αB-crystallin in SJL mice.
Proliferative responses of lymph node cells from mice that were immunized with (A) unphosphorylated αB-crystallin or (B) phosphorylated αB-crystallin. On day 0 and day 7, mice were immunized with 200 µg αB-crystallin in CFA. On day 21 after the first immunization, proliferative responses (stimulation indices) of lymph node cells to unphosphorylated αB-crystallin (open circles), phosphorylated αB-crystallin (closed circles), or Mycobacterium tuberculosis (triangles) were determined in a proliferation assay. Background counts per minute were (A) 1645 cpm and (B) 1851 cpm. On day 19 after immunization with (C) unphosphorylated or (D) phosphorylated αB-crystallin, mice were challenged in the foot with 100 µg of the corresponding protein. Foot swelling (in mm) of individual mice, 24 h and 48 h after challenge, is depicted.

immunization (Figure 4 A and B). As demonstrated before, T cells from SJL mice show virtually no cross-reactivity between the two forms of the peptide. Figure 4 C and D show that modest but clearly detectable DTH responses could also be detected in response to both 41-56 and 41-56P.

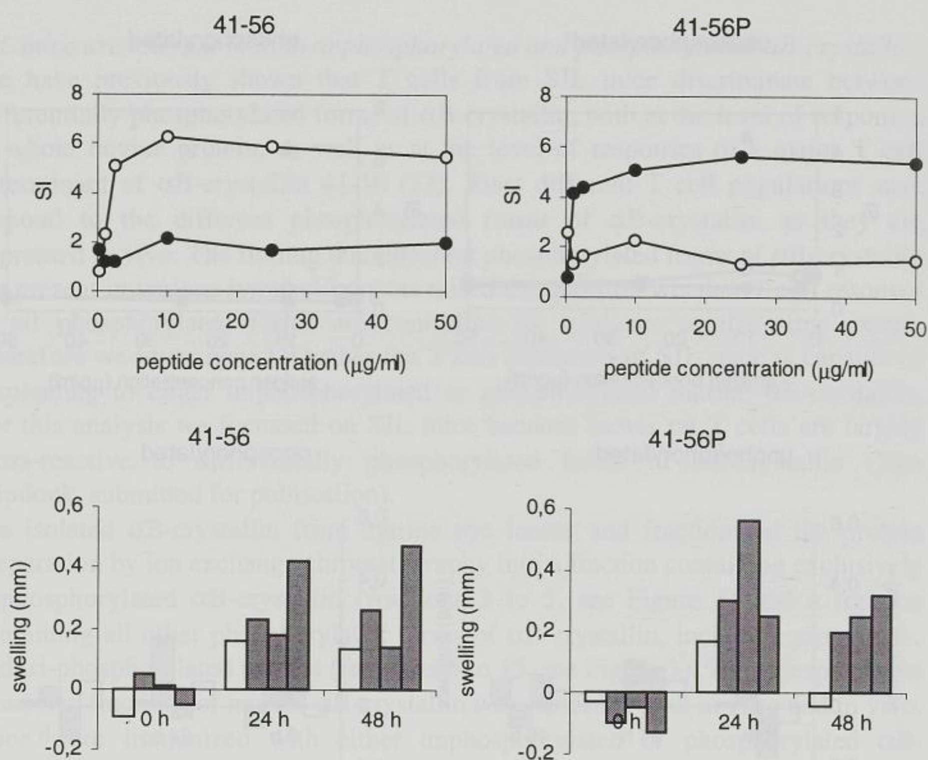

Figure 4. T cell reactivity to both the unphosphorylated and the phosphorylated αB-crystallin epitope 41-56 in SJL mice.
Proliferative responses of lymph node cells from mice that were immunized with (A) 41-56 (B) 41-56P. On day 0 and day 7, mice were immunized with 200 μg αB-crystallin peptide in CFA. On day 21 after the first immunization, proliferative responses (stimulation indices) of lymph node cells to 41-56 (open circles) or 41-56P (closed circles) were determined in a proliferation assay. Background counts per minute were (A) 2722 cpm and (B) 3264 cpm. Stimulation indices to 10 μg/ml Mycobacterium tuberculosis were (A) 13.7 and (B) 11.9. On day 19 after immunization with (C) 41-56 or (D) 41-56P, mice were challenged in the foot with 100 μg of the corresponding peptide. Foot swelling (in mm) of individual mice, 24 h and 48 h after challenge, is depicted.

Discussion

The main findings of this study are that phosphorylation of αB-crystallin varies considerably between different organs of healthy rodents and that T-cell tolerance extends over these differentially phosphorylated forms of αB-crystallin equally well. Thus, while phosphorylation of small stress proteins is generally considered

to be stress-induced, we now show that even in the apparent absence of physiological stress, αB-crystallin can be present in highly phosphorylated forms in some organs. The finding that the murine T cell compartment is tolerant to both unphosphorylated and phosphorylated αB-crystallin corresponds to the presence of these forms of the protein in healthy lymphoid organs.

Expression of αB-crystallin has been demonstrated in rats and mice in several healthy tissues, such as cardiac muscle, skeletal muscle, kidney, spleen, thymus, and brain, but so far no data have been reported on the phosphorylation state of the protein in these organs (17, 18). Recently, Ito et al. showed that various forms of stress induce phosphorylation of αB-crystallin in cardiac muscle (8). In this report we demonstrated that in several untreated tissues of rats and mice, αB-crystallin is present in highly phosphorylated forms. The phosphorylation state of αB-crystallin that we determined is presumably the resultant of phosphorylation by cAMP dependent kinase activity, autokinase activity and dephosphorylation (reviewed by Kantorow and Piatigorsky, 19). Still, the function of phosphorylation of αB-crystallin has not been elucidated. Therefore it is difficult to interpret whether the observed differences in phosphorylation of αB-crystallin in the healthy tissues that we analyzed correlate with differences in function of αB-crystallin in these organs. Notably, we detected highly phosphorylated αB-crystallin in PBMC of both mice and rats. Possibly this results from the constant exposure of PBMC to mechanical stress.

We previously showed that murine T cells can discriminate between differentially phosphorylated forms of αB-crystallin and that phosphorylation may also affect T cell reactivity in a qualitative sense (13, Van Stipdonk et al., submitted for publication). Therefore, in vivo phosphorylation of αB-crystallin may have consequences for an autoimmune response to cells that express the protein. In this report we have demonstrated the presence of different phosphorylated forms of αB-crystallin in healthy primary as well as secondary lymphoid tissues that are involved in tolerance induction. Accordingly, the murine T cell compartment was shown to be tolerant to both unphosphorylated and phosphorylated forms of autologous αB-crystallin. Lewis rat T cells do not clearly discriminate between differentially phosphorylated forms of αB-crystallin (Van Stipdonk et al., submitted for publication). Similar to our findings in SJL mice, T-cell tolerance in Lewis rats applies to all different phosphorylated forms of homologous αB-crystallin, both in vivo and in vitro (data not shown).

Although T cell reactivity to homologous αB-crystallin is tightly controlled, we have shown modest in vivo responses to the major T cell determinant 41-56/41-56P of αB-crystallin. It can not be excluded that under particular conditions, this

determinant may be released from the entire protein by processing and may become presented to T cells. However, we have no indications that 41-56 or 41-56P–specific T cells respond to whole (processed) murine αB-crystallin.
In summary, we have shown in this report that αB-crystallin is present in differentially phosphorylated forms in various organs of rodents in the absence of stress. T cell tolerance is maintained for unphosphorylated as well as phosphorylated αB-crystallin in SJL mice and Lewis rats.

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