# Myocarditis-inducing Epitope of Myosin Binds Constitutively and Stably to I-$A^k$ on Antigen-presenting Cells in the Heart

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## Summary

Immune interactions in the heart were studied using a murine model of myosin-induced autoimmune myocarditis. A T cell hybridoma specific for mouse cardiac myosin was generated from A/J mice and used to demonstrate that endogenous myosin/I-$A^k$ complexes are constitutively expressed on antigen-presenting cells in the heart. This T cell hybridoma, Seu.5, was used as a functional probe to identify a myocarditis-inducing epitope of cardiac myosin. Overlapping peptides based on the cardiac myosin heavy chain α (myhcα) sequences were synthesized and tested for their ability to stimulate Seu.5 T cells. One peptide, myhcα(325–357) strongly stimulated the Seu.5 T cells, localizing the epitope to this region of the myhcα molecule. Using truncated peptides, the epitope was further localized to residues 334–352. The myhcα(334–352) peptide strongly induced myocarditis when administered to A/J mice, which was histologically indistinguishable from that induced by myosin. The myhcα(334–352) epitope was present in cardiac myosin and not skeletal muscle myosins, providing a biochemical basis for the cardiac specificity of this autoimmune disease. Induction of myocarditis by this epitope was restricted to the myhcα isoform and not the myhcβ isoform, suggesting there may be a difference in the efficiency of generating tolerance to these isoforms of cardiac myosin, which are differentially developmentally regulated. The myhcα(334–352) epitope bound to purified I-$A^k$ molecules in a similar manner to other I-$A^k$–restricted immunogenic epitopes, HEL(48–61) and RNase(43–56). Importantly, the myhcα(334–352) epitope was able to bind to I-$A^k$ molecules on the surface of antigen-presenting cells in a stable manner. These findings demonstrate that autoantigenic epitopes can behave in a dominant manner and constitutively bind to class II molecules in the target organ in a similar manner to foreign immunogenic epitopes.

An important property of the immune system is the ability to distinguish self from nonself. Normally this discrimination occurs very efficiently; however, under certain circumstances autoimmune responses develop. Many explanations have been proposed to explain these autoimmune responses, including failures in central or peripheral tolerance, molecular mimicry between a pathogen and a self-antigen, and inadequate regulatory mechanisms (1–3). Despite an intense research effort in this area by numerous laboratories, the cellular and molecular basis for the induction of autoimmune diseases is unknown. The study of autoimmunity has been greatly facilitated by the development of rodent models of autoimmune disease and the identification of the autoantigenic epitopes involved in disease induction (4–7). For example, in the study of experimental autoimmune encephalomyelitis, identification of the disease-inducing epitopes has resulted in a significant advancement in our understanding of autoimmune processes (8–10).

We have a long-standing interest in how the immune system induces cardiac damage, and have used the model of myosin-induced myocarditis to study these interactions (11–13). This model was based on the observation that autoantibodies against cardiac myosin were found in Coxsackievirus B3–infected mice with myocarditis, suggesting cardiac myosin as a candidate autoantigen in autoimmune heart disease (14). Immunization of mice with purified cardiac myosin resulted in the induction of myocarditis with similar pathology to that observed in the Coxsackievirus-infected mice (15). We have previously demonstrated that myosin-induced autoimmune myocarditis is a $CD4^+$ T cell–mediated disease, as depletion of $CD4^+$ T cells prevented induction of myocarditis and transfer of purified myosin-reactive T cells into SCID mice resulted in myocarditis in the absence of anti-myosin antibodies (11). We further explored the requirements for myocarditis induction and used T cell hybridomas specific for cardiac myosin

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to show that processed myosin/I-$A^d$ complexes are expressed on endogenous $Ia^+$ APC in the normal mouse heart (13). Thus, in this model, tissue injury is not required for expression of the autoantigen because the myosin/Ia complexes are constitutively present in the heart.

In this report, we have extended our previous work with myosin-specific T cells, and used these cells as functional probes to identify an epitope of myosin that induces autoimmune myocarditis in mice. We generated a cardiac myosin-specific T cell hybridoma in A/J mice which detected myosin/Ia complexes that were constitutively expressed in the normal A/J heart. We then used a series of overlapping peptides corresponding to the myosin heavy chain α (myhcα)[1] sequence to identify the epitope recognized by this T cell. This myosin epitope induced myocarditis in A/J mice, and bound to I-$A^k$ molecules in an SDS-stable conformation on the surface of APCs. These findings show this is an immunodominant epitope because it is constitutively expressed and stably bound to class II molecules on normal APC in the target organ of this disease model, and suggest that autoantigenic epitopes can be presented in a dominant manner. The stable expression of autoantigen within the target organ in this model of autoimmune disease suggests that the important steps in the induction of myocarditis occur at the level of T cell and/or APC activation.

## Materials and Methods

*T Cell Hybridomas and T Cell Assays.* Myosin-specific T cell hybridomas were generated from myosin-immunized A/J mice as described previously (13). Briefly, A/J mice were immunized subcutaneously with 150 μg of myosin/CFA on days 0 and 7. On day 14 the draining lymph nodes were removed and the cells stimulated in vitro for 7 d with cyanogen bromide–digested mouse cardiac myosin (5 μg/ml) and then restimulated with APC, antigen, and IL-2 for an additional 6 d. The viable cells were then fused to the TCR-$\alpha^-/\beta^-$ BW5147 thymoma cells and myosin-specific T cells were identified by their ability to produce IL-2 upon stimulation with myosin. The reactive T cell hybridomas were subcloned and MHC restriction was determined as previously described (13).

The T cell hybridoma assay was performed as described previously (16). The level of IL-2 production by the T cell hybridomas was ascertained by [$^3$H]thymidine incorporation into the IL-2–dependent CTLL-2 cells.

*Peptides.* Peptides were synthesized, purified, and analyzed as previously described (17). The peptides were synthesized on either an ABI model 432A (Perkin-Elmer, Foster City, CA), or a Symphony/Multiplex synthesizer (Rainin Instrument Co., Woburn, MA) using standard 9-fluorenylmethoxycarbonyl chemistry. The reagents for peptide synthesis were purchased from ABI, Rainin, and Advanced ChemTech (Louisville, KY). The peptides were purified by $C_{18}$ reverse-phase HPLC and their identity confirmed by amino acid analysis (model 6300; Beckman Instruments, Palo Alto, CA) and mass spectrometry (Protein Chemistry Laboratory, Washington University, St. Louis, MO), except the one series of 33 mers which were not HPLC purified. The concentration of each peptide was determined by amino acid analysis.

*Myosin.* Mouse cardiac myosin was purified following a modified procedure of Shiverick et al. (18). The myosin was solubilized and stored in 50 mM sodium pyrophosphate. The cyanogen bromide digest of myosin was prepared as previously described (13).

*APCs.* The CH27 antigen-presenting B cell line (19), cardiac APCs from normal A/J mice (13), and spleen cells (13) were prepared and used as described. Cardiac APCs are bone marrow–derived cells, ~15% of which express MHC class II antigens, with the predominant APC being dendritic cells (20, 21).

*Induction and Quantitation of Autoimmune Myocarditis.* Myocarditis was induced as described previously (11). Briefly, male A/J mice were injected subcutaneously with 100 μg of cardiac myosin emulsified in CFA on days 0 and 7. On day 0 the mice were also injected intraperitoneally with 500 ng of pertussis toxin (List Biochemicals, Campbell, CA). Mice receiving the myhcα peptides were immunized with 50–150 nmol of the peptides emulsified in CFA in an identical manner, and received 500 ng of pertussis toxin as above. Immunization with higher concentrations of the peptides did not result in increased severity of myocarditis. The mice were killed on day 21 and the hearts were prepared for histologic examination. Serial step sections were made through the entire heart and stained with hematoxylin and eosin. Every fifth section was examined for the presence of myocarditis by light microscopy, and was evaluated according to previously published criteria describing the severity of myocarditis as follows: severe = >50% of the heart involved, moderate = 10–50% involved, minimal = <10% involved, or normal (11).

*Peptide Binding to Ia Molecules.* The binding of peptide antigens to purified I-$A^k$ molecules was determined using a competitive binding assay as previously described in detail (22, 23). Briefly, 25 pmol purified I-$A^k$ molecules were incubated with 0.25 pmol $^{125}$I-labeled-HEL(YE52-61) in a total volume of 25 μl in the absence or presence of an unlabeled peptide. A range of concentrations (10–1,000 pmol) of the peptides were tested for their ability to inhibit the binding of the labeled HEL peptide compared to the ability of the unlabeled HEL peptide to inhibit binding to purified I-$A^k$. The level of labeled HEL bound to I-$A^k$ was determined by Bio Spin P6 chromatography (Bio Rad Laboratories, Hercules, CA) (24). In a typical experiment 50,000 to 150,000 cpm of the peptide bound in the absence of competitor peptides, representing 25–40% of the input.

*Ia Stability.* The ability of peptides to induce a stable conformation of I-$A^k$ on the surface of APCs was determined using the SDS/PAGE stability assay as described (23, 25, 26). Briefly, radiolabeled peptides were incubated with the I-$A^k$–expressing M12.C3.F6 B-lymphoma cells for 4 h. The cells were then harvested, washed, and lysed in PBS containing 1% Triton X-100, 10 mM iodoacetamide, 1 mM PMSF, and 20 μg/ml of leupeptin. The lysate was incubated with the monoclonal anti-I-$A^k$ antibody 10.3.6.2 and the immunoprecipitates were analyzed by SDS/PAGE. The SDS stable I-$A^k$ molecules were visualized by autoradiography. The level of I-$A^k$ stability induced was determined by quantitating the stable and unstable bands using a phosphoimage analysis system (Molecular Dynamics, Inc., Sunnyvale, CA).

## Results

*Generation and Characterization of a Myosin-specific T Cell Hybridoma.* T cell hybridomas were generated from A/J

[1] *Abbreviations used in this paper:* MBP, myelin basic protein; mychα, myosin heavy chain α.

mice immunized with autologous cardiac myosin for use as functional probes in the identification of a myocarditis-inducing epitope. One T cell hybridoma, Seu.5, was selected for further characterization. Seu.5 strongly reacted to murine cardiac myosin (Fig. 1 *A*). This hybridoma was CD4 dependent, did not respond to actin, troponin, tropomyosin, or myosin light chain, and was restricted by the I-$A^k$ molecule (data not shown). Previous studies demonstrated that myocarditis in A/J mice was an I-$A^k$–mediated disease (11). Thus, we had generated a cardiac myosin-specific T cell hybridoma, specific for the autoantigen and restricted by the appropriate MHC molecule, which we could use as a probe to identify an epitope involved in the induction of myocarditis.

We have previously shown that cardiac myosin is constitutively processed and presented by cardiac APCs using an I-$A^d$–restricted myosin-specific T cell hybridoma (13). We next determined if the epitope recognized by Seu.5 was also processed and presented in the hearts of normal A/J mice. As shown in Fig. 1 *B*, residential A/J cardiac APCs strongly stimulated Seu.5 T cells. The expression of the myosin–I-$A^k$ complexes was tissue specific because no complexes were detected by Seu.5 T cells in the spleens from the same animals (Fig. 1 *C*). These findings indicated that the myosin epitope recognized by Seu.5 is constitutively processed and presented in the target organ of this model of autoimmune myocarditis.

*Identification of the Region of Myosin Recognized by Seu.5.* To identify the epitope of myosin recognized by Seu.5, we synthesized a series of peptides based on the A/J murine cardiac myhcα isoform sequence (27). None of the peptides in a series of overlapping peptides covering residues 1–149 stimulated Seu.5 (data not shown). We then synthesized 24 peptides encompassing residues 145–637 of the myhcα sequence, each 33 amino acids long with overlaps of 13 amino acid residues. Each of the peptides was then tested for its ability to stimulate Seu.5 and the results are shown in Fig. 2. One of the peptides, myhcα(325–357), strongly stimulated Seu.5, whereas none of the others stimulated Seu.5. Thus, the myhcα epitope recognized by Seu.5 was contained within the peptide 325–357 (Fig. 3 *A*).

*Characterization of the myhcα(325–357) Determinant.* To identify the actual Seu.5 stimulatory determinant, we generated a series of truncated peptides based upon the myhcα(325–357) sequence (Fig. 3 *A*). The inability of the overlapping peptides myhcα(305–337) and myhcα(345–377) to stimulate Seu.5 (Fig. 2), suggested that the stimulatory epitope was not contained in the amino or carboxy terminal regions of the myhcα(325–357) sequence. Therefore, we initially focused on the central portion of the 325–357 epitope by synthesizing the peptide myhcα(334–352). The myhcα(334–352) peptide stimulated Seu.5 T cells as strongly as the myhcα(325–357), indicating that the epitope was contained within this 19 mer (Fig. 3 *B*). The optimal length of class II–restricted epitopes varies, but is generally 13–15 amino acids. To identify the optimal length of the Seu.5 epitope, we generated truncated peptides of the myhcα(334–352) sequence (Fig. 3 *A*). The 14 mer, myhcα(335–348) peptide strongly stimulated Seu.5 T cells, whereas the same length peptide shifted one amino acid in register, myhcα(334–347), was completely nonstimulatory (Fig. 3 *B*). This failure to stimulate appeared to be due to the lack of the A348 residue, since the peptide myhcα (334–348) was strongly stimulatory. Thus, we had localized the Seu.5 stimulatory myhcα epitope to the 14–amino acid peptide myhcα(335–348).

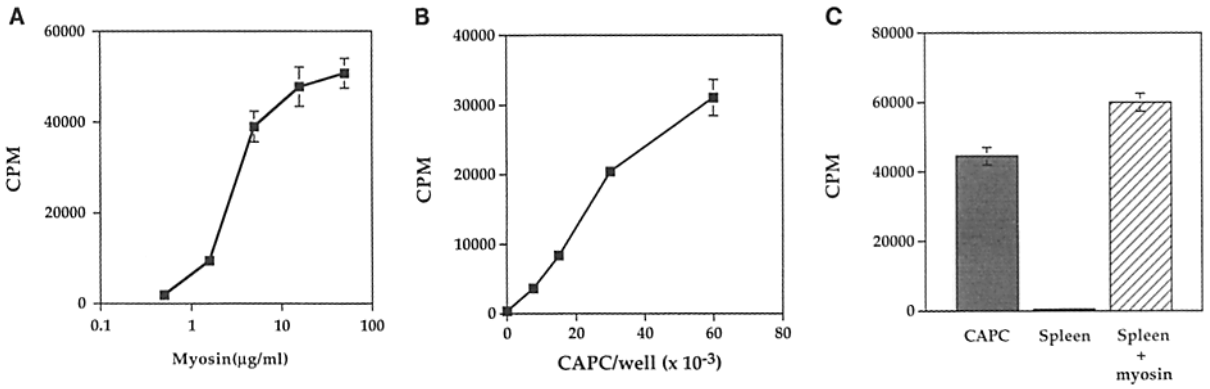


**Figure 1.** Reactivity of Seu.5 T cell hybridoma. (*A*) Seu.5 recognizes mouse cardiac myosin. Seu.5 T cells ($10^5$) were stimulated for 20 h with CNBr-digested mouse cardiac myosin at the indicated concentrations using CH27 cells ($3 \times 10^4$) as the APC as described in Materials and Methods. The level of stimulation was determined by quantitating the level of IL-2 production by [$^3$H]thymidine incorporation in the IL-2–dependent cell line, CTLL-2. Stimulation by APC without any antigen resulted in <200 cpm. The values represent the mean +/− standard deviation of triplicate determinations. Identical results were obtained in at least three separate experiments. (*B*) Seu.5 recognizes myosin/I-$A^k$ complexes expressed constitutively on cardiac APCs. Seu.5 cells were stimulated with increasing numbers of purified cardiac APCs (*CAPC*) from A/J mice without the addition of exogenous antigen as described in *A*. The values represent the mean +/− standard deviation of triplicate determinations. Identical results were obtained in three separate experiments. (*C*) Endogenous myosin/I-$A^k$ complexes are expressed in the heart but not in the spleen. Seu.5 T cells ($10^5$) were stimulated with (*1*) A/J CAPC ($2 \times 10^4$/well) with no additional antigen, (*2*) spleen cells ($5 \times 10^5$) from the same mice alone, or (*3*) with the addition of 10 μg/ml of CNBr-digested mouse cardiac myosin. The values represent the mean +/− standard deviation of triplicate determinations. Identical results were obtained in three separate experiments.

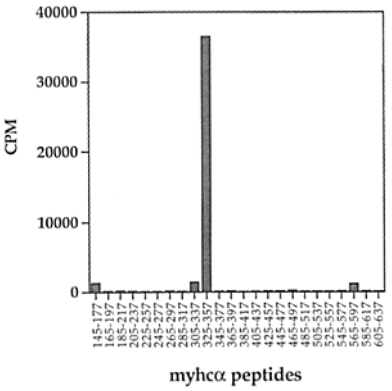


**Figure 2.** Seu.5 T cells recognize myhcα(325–357). Seu.5 T cells ($10^5$) were stimulated with CH27 ($3 \times 10^4$) APC and 0.5, 5, and 50 μM of each of a series of 24 overlapping peptides covering the myhcα residues 145–637 as described in Fig. 1. The amino acids covered by each peptide are given based on the myhcα sequence. The values shown are from the 50-μM concentration. An identical pattern of reactivity of the peptides was observed at the other two concentrations tested. The values represent the mean of triplicate determinations. The standard deviations were <15% of the mean. Similar results were obtained in two separate experiments. The very weak but detectable responses observed for 145–177, 305–337, and 565–597 peptides were not observed in any other experiments, even when tested at higher concentrations.

*myhcα(334–352) Induces Myocarditis.* Having identified the myosin epitope recognized by the Seu.5 T cells, we next wanted to determine if this epitope could induce myocarditis. When A/J mice were immunized with the myhcα(334–352) peptide, 11 of 11 mice developed myocarditis in three separate experiments (Fig. 4 *A*). The frequency and severity of myocarditis induced by myhcα (334–352) was similar to native myosin and the inflammatory lesions appeared histologically similar (Fig. 4 *B*), although neutrophils were more common in myosin-induced disease than in peptide-induced disease. With the large size of myhcα, it would be surprising if this disease was restricted to a single epitope. However, these data indicate that myhcα-(335–348) is a major epitope of myosin involved in autoimmune myocarditis because immunization with this peptide induced significant myocarditis. Thus, we have identified an epitope of the α isoform of cardiac myosin which is constitutively expressed on cardiac APC and can induce autoimmune myocarditis.

*The myhcα(334–352) Determinant Is Cardiac Specific.* Autoimmune myocarditis is a cardiac-specific disease as immunization with cardiac myosin results in myocarditis, and not skeletal muscle myositis (15). Conversely, immunization with skeletal myosin induces myositis, but no cardiac inflammation (28). One explanation for this tissue specificity is that the myocarditis-inducing epitope is expressed on cardiac myosins and not skeletal muscle myosins. To ascertain if this possibility was correct, we compared the myhcα-(335–348) sequence to the corresponding region of other cardiac and skeletal myosins. Vertebrate myosins are part of a multigene family comprised of at least 10 different members, all of which have a conserved structure (29). Protein sequence alignments of the myosins revealed areas of complete conservation and others that are highly divergent. The 334–352 region of myosin is not conserved between different myosin molecules (Fig. 5 *A*). Several positions in rat, human, and mouse myosins differ in this region, including both cardiac and skeletal myosins (Fig. 5 *A* and not shown). The 334–352 region was identical between the three described allelic forms of the mouse myhcα gene (15, 27), consistent with them all being able to induce myocarditis (11). Thus, the observed cardiac specificity of this autoimmune disease can be explained by the myhcα(334–352) sequence only being expressed in the cardiac isoform of myosin heavy chain.

*Seu.5 Specifically Recognizes the myhcα Isoform of the (334–352) Epitope.* Two isoforms of the cardiac-specific myosin heavy chains are expressed in mammals, myhcα, and myhcβ. The myhcα(334–352) determinant differed from the murine and rat myhc-β sequences at positions 341, 344, and 348–350, whereas rat myhcα differed only at po-

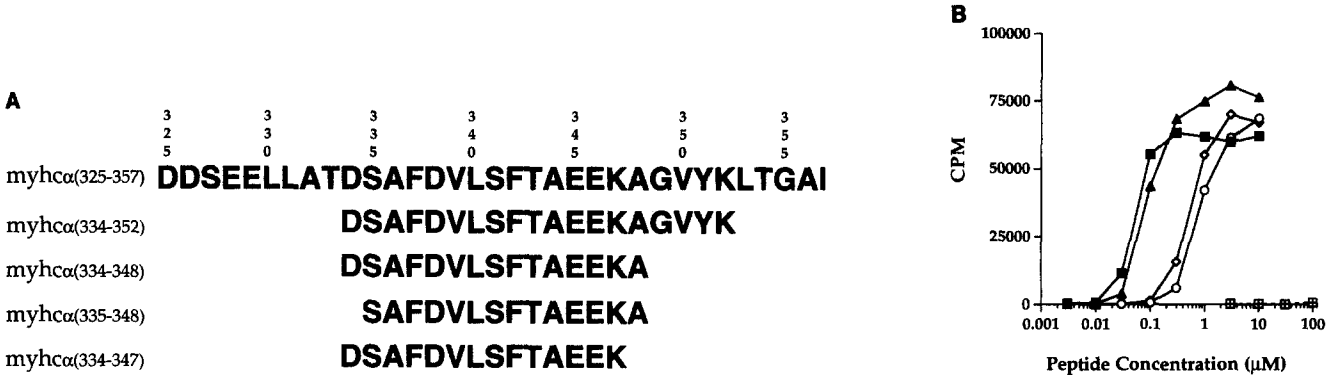


**Figure 3.** Seu.5 reactivity to myhcα(325–357) and truncated peptides. (*A*) Sequence of myhcα(325–357) and nested peptides. The amino acid sequence of myhcα(325–357) and four shorter peptides are shown using the standard one-letter amino acid code. (*B*) Stimulation of Seu.5 by myhcα(325–357) and truncated peptides. Seu.5 T cells ($10^5$) were stimulated with CH27 ($3 \times 10^4$) APC and the indicated concentrations of each of the peptides shown in *A* as described in Fig. 1 *A*. The values represent the mean +/− standard deviation of triplicate determinations. Identical results were obtained in at least four separate experiments. ▲, myhcα(325–357); ■, myhcα(334–352); ○, myhcα(334–348); ◇, myhcα(335–348); ⊞, myhcα(334–347).

A

| Experiment | Antigen | Total | Severity of Myocarditis | | | |
|---|---|---|---|---|---|---|
| | | | Normal | Minimal | Moderate | Severe |
| 1 | myhcα(334-352) | 5 | 0 | 1 | 2 | 2 |
| 1 | myosin | 3 | 1 | 1 | 1 | 0 |
| 2 | myhcα(334-352) | 3 | 0 | 3 | 0 | 0 |
| 2 | myhcβ(334-352) | 3 | 3 | 0 | 0 | 0 |
| 3 | myhcα(334-352) | 3 | 0 | 1 | 1 | 1 |
| 3 | myhcβ(334-352) | 3 | 3 | 0 | 0 | 0 |
| 3 | myosin | 2 | 0 | 0 | 0 | 2 |

B

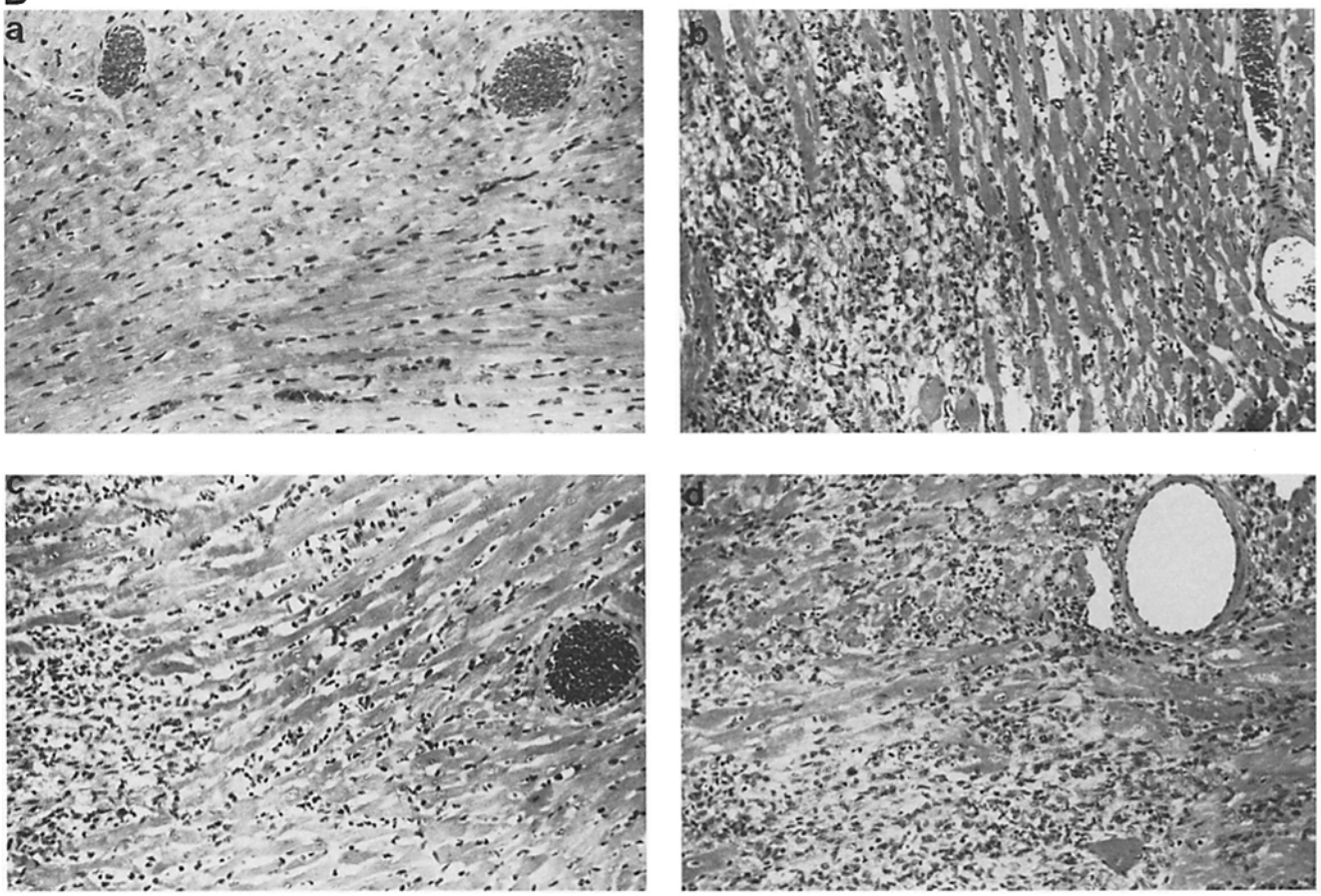


**Figure 4.** myhcα(334–352) induces myocarditis in A/J mice. (*A*) Summary of the severity of myocarditis induced by myhcα(334–352) in three separate experiments. A/J mice were immunized with 50–100 nmol of myhcα(334–352), myhcβ(334–352), or cardiac myosin and the degree of inflammation in the hearts was determined as described in Materials and Methods. (*B*) Photomicrographs of cross-sections of hearts stained with hematoxylin and eosin obtained from mice from experiment 1 (Fig. 4 *A*) immunized with either 100 μg of myosin (*a* and *b*), or 100 nmol myhcα(334–352) (*c* and *d*). All magnifications are 200. (*a*) Normal, myosin-immunized. (*b*) Moderate myocarditis, myosin immunized. (*c*) Moderate myocarditis, myhcα(334–352) immunized. (*d*) Severe myocarditis, myhcα(334–352) immunized. Estimates of the severity of myocarditis are made based on examination of the entire cross-sectional area, as described in Materials and Methods.

sition 341 (Fig. 5 *A*) (30). To ascertain if these sequence variations affected the immunogenicity of the myhcα-(334–352) epitope, we generated the corresponding determinant from the myhcβ sequence. The myhcβ(335–348) peptide did not stimulate the Seu.5 T cells at concentrations as high as 100 μM (Fig. 5 *B*). Similarly, the myhcβ-(334–352) peptide did not stimulate Seu.5 T cells (data not shown). These findings demonstrated that Seu.5 specifically recognized the myhcα(334–352) isoform. To identify which of the amino acid differences between these two se-

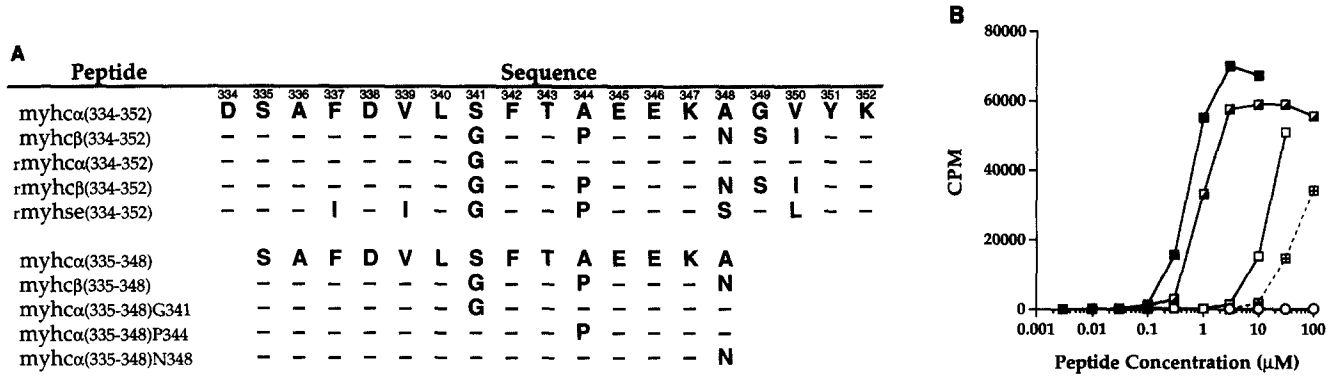


**Figure 5.** Fine specificity of the myhcα(334–352) epitope. (*A*) Comparison of myhcα(334–352) to other myosins. The sequence of myhcα(334–352) epitope compared to the corresponding region in other myosin molecules (*upper half*) and myhcα(335–348) and related peptides (*lower half*) are shown using standard one-letter amino acid codes. The "–" indicates identity to the myhcα(334–352) sequence with the amino acid differences being shown. myhcα, mouse cardiac myosin heavy chain α isoform (27); myhcβ, mouse cardiac myosin heavy chain β isoform (Beisel, K.W., personal communication); rmyhcα, rat cardiac myosin heavy chain α isoform (30); myhcβ, rat cardiac myosin heavy chain β isoform (30); rmyhse, rat embryonic skeletal muscle myosin isoform (40). Peptides containing substituted amino acids are designated by the position and the substituted residue. (*B*) Seu.5 stimulation by myhcα(334–352) and related peptides. Seu.5 T cells ($10^5$) were stimulated with CH27 ($3 \times 10^4$) APC and various concentrations of the indicated peptides shown in *A*, as described in Fig. 1 *A*. The values represent the mean of triplicate determinations with the standard deviations not exceeding 15% of the mean. Identical results were obtained in three separate experiments. ■, myhcα(335–348); ○, myhcβ(335–348); ◪, myhcα(335–348)G341; □, myhcα(335–348)P344; ⊞, myhcα(335–348)N348.

quences were responsible for this specificity, singly substituted peptides were generated (Fig. 5 *A*). We used the myhcα(335–348) sequence for this analysis because it was fully stimulatory, thereby eliminating positions 349 and 350 from study. Of the three remaining positions which differed between the myhcα and myhcβ sequences, two of the residues, A344 and A348, appeared to be involved in this isoform specificity. Substitution at either position with the corresponding myhcβ residue significantly decreased the ability of the peptides to stimulate Seu.5 T cells (Fig. 5 *B*), with A344 being ~30-fold and A348 being 100-fold less active. The S341 residue did not appear to be a critical residue because its replacement with a G did not significantly affect reactivity (Fig. 5 *B*). These findings indicated that the recognition of the myhcα(334–352) determinant critically involved both P344 and N348 residues.

*Myocarditis Induction by myhcα(334–352) Is Restricted to the myhcα Isoform.* The Seu.5 T cell specifically recognized the myhcα(334–352) isoform of the determinant. We next ascertained if this same specificity was observed in myocarditis induction. When A/J mice were immunized with either the myhcα(334–352) or the myhcβ(334–352) peptides, we found that the myhcβ(334–352) peptide did not induce myocarditis, whereas the myhcα(334–352) strongly induced myocarditis (Fig. 4 *A*). Therefore, the induction of myocarditis was specific to the myhcα isoform of the 334–352 determinant.

*myhcα(335–348) Epitope Binds Stably to IA$^k$ Molecules.* The ability of autoimmune disease–inducing epitopes to bind to MHC molecules has been demonstrated for only a limited number of epitopes. Studies involving the extensively characterized MBP(Ac1-11) epitope showed that it bound very weakly to the I-A$^u$ molecule, even though it could strongly induce experimental autoimmune encephalomyelitis (31). A study of the binding of autoimmune disease–related epitopes in Lewis rats revealed that the epitopes were intermediate to poor binders (32). We wanted to ascertain how strongly the myhcα(334–352) epitope bound to the IA$^k$ molecules. There have been many I-A$^k$–restricted immunogenic peptides identified (33). Sequence comparison of the myhcα(334–352) epitope with a panel of I-A$^k$–restricted epitopes showed homology to the extensively characterized HEL(48–61) determinant. This homology was in the I-A$^k$–binding residues, D338 and K348, which correlated with D52 and R61 residues of HEL(48–61), respectively. Our previously shown data demonstrated that myhcα(334–352) stimulated the I-A$^k$–restricted Seu.5 T cell (Fig. 2). To ascertain how avidly the myhcα(334–352) epitope bound to the I-A$^k$ molecule, a direct binding assay was performed (Fig. 6 *A*). These studies revealed that the myhcα(334–352) epitope bound strongly to I-A$^k$. This binding was stronger than shown for the immunodominant I-A$^k$–restricted epitope, bovine RNase(43–56) (17), but was weaker than the canonical I-A$^k$ binder, HEL(48–62) (22, 23). Thus, in contrast to the MBP(Ac1-11), the myhcα(335–348) epitope bound strongly to its restriction element.

Immunodominant I-A$^k$–binding peptides induce a conformational stability upon the I-A$^k$ molecule which can be determined by SDS/PAGE analysis. This stability directly correlates with the half-life of the peptide binding to the I-A$^k$ molecule on the surface of APCs and to their immunogenicity. Thus, immunodominant determinants of antigens induce the SDS-stable conformation of I-A$^k$ and have long half-lives on the surface of APC. The myhcα(335–348) determinant contained a D residue at position 338, which would be predicted to facilitate stable I-A$^k$ formation (34). The myhcα(334–352) peptide was radiolabeled

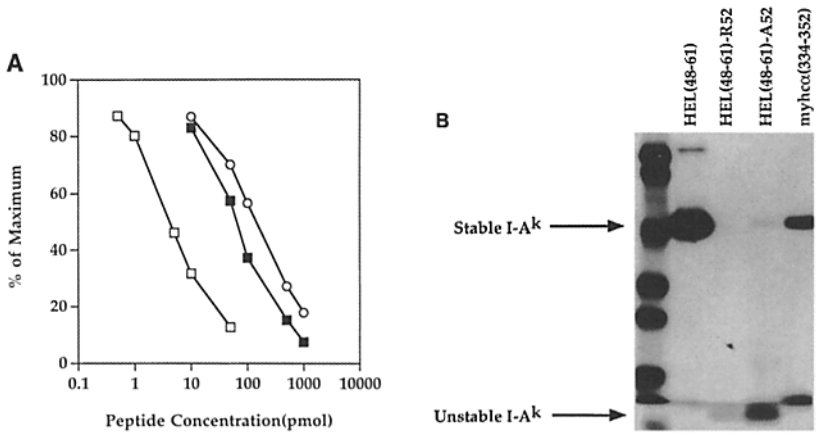


**Figure 6.** Binding of myhcα(334–352) to I-$A^k$. (*A*) Binding of myhcα(334–352) to purified I-$A^k$ molecules using a competitive binding assay as described in Materials and Methods. The values represent the percent maximum cpm bound of the HEL(YE52-61) peptide to I-$A^k$ in the presence of the indicated concentrations of unlabeled HEL(YE52-61) (YEDYGILQINSR), myhcα(334-352) (DSAFDVLSFTAEEKAGVYK), or RNase(43–56) (VNTFVHESLADVQA). The maximum binding in the absence of competitors was 148,469 cpm. The values represent a single determination. The relative $IC_{50}$ values of the three different peptides were HEL(YE52-61) = 1.0, myhcα(334–352) = 15.6, and RNase(43–56) = 33.3. These results were confirmed in two separate experiments. □, HEL(46–61); ■, myhcα(334–352); ○, RNase(43–56). (*B*) myhcα(334–352) induces an SDS-stable conformation of I-$A^k$. An autoradiogram of a 10% SDS-PAGE gel of lysates from M13.C3.F6 cells incubated for 4 h with $^{125}$I-labeled HEL(48–61) (DGSTDYGILQINSR) which induced stable I-$A^k$ formation, HEL (48–61)-R52(DGSTRYGILQINSR) and HEL (48–61)-A52 (DGSTAYGILQINSR) unstable inducers, or myhcα(334–352). The expected position of the SDS stable intact α/β heterodimer and the unstable I-$A^k$ molecules are indicated. The left-most lane contained molecular mass markers of 106,000, 80,000, 49,500, 32,500, 27,500, and 18,500 kD. Identical results were obtained in two individual experiments.

on the Tyr350 residue and tested for its ability to induce the SDS-stable conformation of the I-$A^k$ molecules on the surface of live I-$A^k$–expressing APCs. The myhcα(334–352) induced the formation of SDS-stable I-$A^k$ molecules (Fig. 6 *B*). The level of stable I-$A^k$ was 50%, demonstrating a propensity of myhcα(334–352) to favor stable conformations of the I-$A^k$ molecules. This level of stability was less than the strong stable inducer, HEL(48–61) (96% stable), but significantly higher than unstable variants of HEL(48–61) (21% stable). These findings showed that the myhcα-(335–348) determinant bound to I-$A^k$ with a good affinity and with a reasonable propensity to form a stable conformation. When APC were pulsed with the myhcα(335–348) peptide in tissue culture assays, the Seu.5 T cells were strongly stimulated (data not shown), providing additional support of the mychα(334–352) epitope behaving as a dominant epitope. These findings are also consistent with our biological observations that this determinant is constitutively expressed on APC in the heart.

## Discussion

In these studies we identified an autoantigenic epitope of mouse cardiac myosin heavy chain, myhcα(334–352). This epitope induced myocarditis comparable to that induced by native cardiac myosin, indicating that it was the major autoantigenic determinant of myhcα in A/J mice. The basis for the cardiac specificity of autoimmune myocarditis was provided by the sequence of the myhcα(334–352) determinant, being uniquely expressed in myhcα and not in the myhcβ or in skeletal muscle myosins. The myhcα(334–352) epitope bound strongly to purified I-$A^k$ and had a good propensity to form stable complexes on the surface of live APCs.

The steps involved in the induction of an autoimmune disease are complicated and most likely heterogeneous in nature. Simplistic views of the normal mechanisms involved in preventing autoimmune disease involve the absence of a key component, such as the T cells or the antigen. Thus, under normal situations the autoreactive T cells would not exist, and some type of failure in tolerance would allow these T cells to persist. Our studies have clearly shown that myosin-specific autoreactive T cells are present in normal animals, thus the failure of normal mice to spontaneously develop myocarditis cannot be due to a lack of the autoreactive T cells. Prevention of autoimmune reactions by sequestering the autoantigen from the immune system is another related possibility. This sequestration model contends that tissue injury, release of the self antigen, and subsequent exposure to the immune system would result in autoimmunity. This mechanism could be operating for antigens such as MBP which are sequestered by the blood-brain barrier. However, the demonstration that a precursor of MBP is expressed in the thymus complicates the interpretation for the existence of MBP-reactive T cells (35). Sercarz and his colleagues have proposed that autoantigenic epitopes are part of the cryptic self repertoire and that dominant self epitopes induce tolerance (36).

In autoimmune myocarditis, we have convincingly shown that inaccessibility of the autoantigen to the immune system or being part of the cryptic self repertoire cannot be the explanation for the lack of stimulation of the myosin-reactive T cells. Our previous studies clearly demonstrated that cardiac myosin was constitutively processed and presented on APCs in the heart. In the work presented in this report, we have identified an epitope of myosin that can induce myocarditis by using an I-$A^k$–restricted myosin-specific T cell hybridoma as a functional probe. Though we do not have direct evidence that this hybridoma came from a T cell clone capable of inducing myocarditis, we believe Seu.5 is representative of a pool of myocarditis-inducing T cells because its stimulating epitope myhcα(334–352) is

constitutively expressed on cardiac APC and causes myocarditis when injected into A/J mice. We have shown that the myocarditis-inducing epitope, myhcα(334–352), binds strongly to I-$A^k$ molecules and induces a stable conformation. This stable conformation of I-$A^k$ has been previously shown to result in long-lived complexes on the surface of APC, and correlates directly with the immunodominance of the epitope. The finding that 334–352 induces stable I-$A^k$ molecules supports our observation that the myhcα-(334–352)/I-$A^k$ complexes are expressed constitutively on APC in the heart.

These results indicate that autoimmune disease–inducing epitopes can bind strongly and stably to the MHC molecules. Thus, the myhcα(334–352) epitope is constitutively accessible to the immune system in an immunodominant manner, and the mechanism preventing myocarditis in normal mice is not due to either the lack of myosin-specific T cells or the availability of the myhcα(334–352) epitope. Therefore, the view that the prevention of autoimmune reactions is due to the absence of either the T cells or available antigen appears to be too simplistic with regard to autoimmune myocarditis. Our findings would clearly indicate that it must be the activation state of the T cells, the APC, or the local environment in the heart, which normally prevents autoimmunity in the heart.

The identification of the myhcα(334–352) epitope as a dominant myocarditis-inducing epitope permitted further characterization of the myosin-induced myocarditis model. These findings clearly demonstrate that the disease-inducing autoantigen is cardiac myosin. The cardiac specificity of this disease can also be explained because the myhcα(334–352) epitope is only found in cardiac myosin, not in skeletal muscle myosins, and the constitutively expressed complexes are not found in other APCs outside of the heart. Interestingly, myocarditis induction by myhcα(334–352) was specific for the myhcα isoform, and not the β isoform. In rodents the β isoform predominates in the heart until birth, at which time the α isoform then becomes the dominant cardiac myhc isoform. After birth, the β isoform is expressed in the ventricles of the heart as well as in other noncardiac muscles. A simple explanation for our finding that induction of myocarditis is caused by the myhcα epitope, would be that this area of the myosin molecule differs significantly between isoforms of myosin, including other muscle myosins and cardiac β isoform. The observed myhcα specificity may not be due to differences in the β cardiac isoform per se, but may simply reflect that the myhcα(334–352) epitope differs from the other myosins, whereas the myhcβ(334–352) determinant is similar to all of the other forms of myosin. Thus, the mice would be tolerant in this region to other forms of myosin, except myhcα. Another more interesting possibility would be that somehow there is a difference in the level of tolerance to the different myhc isoforms. There could be more complete tolerance to the β isoform than the α, resulting in the deletion of the self-reactive T cells which could recognize the β isoform. Perhaps, the recognition of the β isoform during fetal and neonatal life by the developing immune system results in more efficient tolerance induction, because the α isoform is not expressed until after birth. Thus, this would be an example of a tolerance being affected by a developmentally expressed self-antigen. Obviously further studies must be performed to determine if tolerance due to differential developmental expression of the α and β cardiac myosin isoforms is directly related to the disease process.

The myhcα(334–352) epitope, located in the S1 head region, has not been previously implicated in any immunological studies involving cardiac myosin. Wegmann et al. identified two myocarditogenic epitopes in rats, comprised of residues 1304–1320 and 1539–1555 (37). Liao et al. using bacterially expressed fragments of rat cardiac myosin heavy chains, concluded that some of the pathogenic epitopes of myosin resided in the S2 subunit, residues 736–1032 in BALB/c mice (38). The myhcα(334–352) epitope identified in this report represents an immunodominant autoantigenic T cell epitope in A/J mice and most likely in other I-$A^k$–expressing strains; however, other epitopes of myosin are likely involved in this disease in strains of mice possessing different MHC alleles.

A key finding from this work is that autoantigenic epitopes can behave like immunodominant epitopes, bind strongly to the class II molecules, and remain stably expressed on the surface of an APC. Sette and colleagues (39) and Buus and colleagues (32) have made similar conclusions that autoantigenic epitopes do not necessarily have to be poor binders. These findings suggest that there is heterogeneity in the biochemical nature of autoantigenic epitopes with myhcα(334–352) representing one group, and the MBP Ac1-11 representing another. The difference in stability of these epitopes binding to their respective restriction elements could indicate that different mechanisms may be responsible for the self-reactive T cells in the different autoimmune diseases. It is intriguing to speculate that the mechanism involved in myocarditis induction would be the developmental expression of the different isoforms of the cardiac myosin heavy chain, with the myhcα, which contains the disease inducing epitope, not being expressed until birth and only in the heart.

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