Identification of an Autologous Insulin B Chain Peptide as a Target Antigen for H-2K^b-restricted Cytotoxic T Lymphocytes

By James M. Sheil,* Sara E. Shepherd,* Gerald F. Klimo,* and Yvonne Paterson‡

*From the *Department of Microbiology and Immunology, West Virginia University Health Sciences Center, Morgantown, West Virginia 26506; and the ‡Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Summary

We have examined the CD8$^+$ peripheral T cell repertoire of C57BL/6 (H-2^b) mice for cytotoxic T lymphocyte (CTL) reactivities to insulin, using in vitro immunization with a chymotryptic digest of reduced bovine insulin. The results presented in this study demonstrate that potentially autoreactive H-2K^b-restricted cytotoxic T cells specific for an autologous insulin B chain peptide are present in the preimmune splenic T cell repertoire. The immunogenic peptide comprises residues 7–15 from the insulin B chain and has features in common with naturally processed K^b-restricted peptides identified by others. The minimal peptide sequence recognized by these cytotoxic T cells is 10–15, which is highly conserved in mammalian species and constitutes a self-peptide in mice. The presence of class I major histocompatibility complex–restricted CTLs with potentially autoreactive specificities in preimmune animals raises the possibility of a role for such cells in autoimmune disease states. Possible mechanisms for the in vivo expansion of insulin peptide-specific CTLs are discussed.

Insulin has been studied extensively by immunologists as a model antigen system for examining the genetic and cellular regulation of Th cell recognition in class II MHC-restricted cell-mediated immune responses. These investigations have provided significant insight into the processing requirements and structural constraints involved in generating antigenic peptide fragments in the class II MHC presentation pathway. A number of studies have demonstrated that maintenance of an intact interchain disulfide linkage between the A and B chains is necessary for the effective Th response to several dominant insulin epitopes (1–5). However, other investigators have shown that a class II H-2-restricted Th response can be induced against both insulin A chain– and insulin B chain–derived peptides in the absence of the disulfide-linked B and A chain fragments, respectively (6–9). It is tempting to speculate that the Th response to one or more of these insulin-derived peptides may be relevant to the pathogenesis of insulin-dependent (type I) diabetes mellitus (IDDM).[1] In this regard, the well-established association between expression of certain class II MHC alleles and susceptibility to IDDM has focused the interests of immunologists on the possible role of class II MHC-restricted, CD4$^+$ Th cells in the pathogenesis of this autoimmune state (10). Indeed, substantial evidence has been obtained that implicates CD4$^+$ Th cell involvement as a possible autoimmune effector mechanism in the progression of IDDM (11–14), although the target autoantigen in IDDM remains unidentified.

Several reports, however, have suggested a similar involvement of CD8$^+$, class I MHC-restricted CTLs in the pathogenesis of IDDM (15–17). It has also been speculated that a likely target antigen for such autoimmune CTLs may be a self-peptide derived from processing of the insulin molecule in the class I presentation pathway (18). With our current understanding that class I MHC-restricted CTL responses also require antigen processing for effective presentation to CD8$^+$ CTLs, a large effort is presently underway to more fully understand the processing requirements and structural characteristics for antigenic peptides generated in the class I MHC presentation pathway. An in vitro approach designed to induce CTLs specific for peptides of defined sequence has been introduced by Bevan and co-workers (19), whereby the in vitro stimulation of spleen cells with a complete tryptic digest of ovalbumin was employed for the induction of peptide-specific, CD8$^+$ CTLs. In the present study we have employed a modified form of this approach to obtain class I H-2^b-restricted, CD8$^+$ CTLs specific for an insulin-derived peptide. In addition to providing information concerning the structural basis for insulin peptides as antigens for class I MHC-

[1] *Abbreviations used in this paper:* BI, bovine insulin; IDDM, insulin-dependent diabetes mellitus; SN, supernatant; VSV N, vesicular stomatis virus nuclear (protein peptide).

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/02/0545/08 $2.00
Volume 175 February 1992 545–552

restricted CTL recognition, identification of CTL-recognized insulin peptides might contribute to our understanding of the proposed involvement of $CD8^+$ CTLs as autoimmune effector cells involved in the pathogenesis of IDDM.

One prerequisite for such a role for $CD8^+$, insulin peptide-specific CTLs is to demonstrate that such potentially autoreactive T cells are present in the preimmune repertoire of normal animals. In this report we describe the induction of $CD8^+$, insulin peptide-specific CTLs by in vitro stimulation of C57BL/6 ($H\text{-}2^b$) spleen cells with a chymotryptic digest of reduced bovine insulin (BI). The characterization of these B6-derived CTLs indicates they are specific for an autologous insulin B chain peptide and further demonstrates the existence of such autoreactive CTLs in the spleen-derived mature T cell repertoire of naive C57BL/6 mice. Possible mechanisms for the maintenance of these potentially autoreactive CTLs, without any apparent detrimental effects, in the preimmune repertoire are considered.

Materials and Methods

Animal. All mice used in this study were originally obtained from either The Jackson Laboratory (Bar Harbor, ME) or Scripps Clinic and Research Foundation (La Jolla, CA) and have been subsequently bred and maintained in microisolator cages in our colony at the West Virginia University Health Sciences Center Vivarium. Mice were routinely used for experimental studies between 8 and 12 wk of age. Female rats of the Lewis strain were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and used, at ~10 wk of age, for the preparation of rat spleen cell-derived Con A supernatant (SN).

Cell Lines. All cell lines are maintained by serial passage in RP-10 media (RPMI 1640 plus 10% heat-inactivated FCS and 1% L-glutamine, penicillin/streptomycin, and 5×10^{-5} M 2-ME). EL4 is a thymoma-derived tumor cell line from the C57BL/6 ($H\text{-}2^b$) mouse strain. P815 is a mastocytoma-derived tumor cell line from the BALB/c ($H\text{-}2^d$) strain, and the CBA.D1 tumor cell line is derived from the CBA ($H\text{-}2^k$) strain. The transfected cell lines, $L+K^b$ and $L+D^b$, are derived from the $LMTK^-$ ($H\text{-}2^k$) fibroblast cell line and were a generous gift from Dr. Stanley G. Nathenson (Albert Einstein College of Medicine, Bronx, NY).

Antigens. BI and purified A and B chains were purchased from Sigma Chemical Co. (St. Louis, MO). The denatured form of BI was prepared by incubation, with stirring overnight of a 10-mg/ml solution in 8 M urea plus 200 mM 2-ME in 0.1 M Tris buffer, pH 8.3. Chymotryptic digestion was carried out by addition of 5% (wt/wt) α-chymotrypsin (Sigma Chemical Co.) to either denatured or native insulin, or purified A or B chain, in buffer, pH 8.3. The digestion with chymotrypsin was allowed to proceed, with stirring, for 24 h at 37°C.

Individual HPLC-derived fractions were obtained after reverse-phase HPLC analysis of the whole chymotryptic digest on a semi-preparative C-18 (octadecylsilane) column (Vydac; The Separations Group, Hesperia, CA). Fractions were eluted at 2 ml/min with a linear gradient of 0–50% acetonitrile, over a time course of 90 min. Peptides were synthesized as previously described (20, 21) using a standard manual solid-phase synthesis procedure on a polystyrene-co-1% divinylbenzene resin and tert-butyloxycarbonyl for all N^{α}-protection of amino acids. Couplings were carried out using *N-N''*-diisopropylcarbodiimide and were monitored by ninhydrin reaction (22). Simultaneous resin cleavage and side-chain deprotection was achieved by the high-low hydrogen fluoride method (23). The crude products were purified to 98% purity by reverse-phase HPLC as previously described (20, 21), and peptide compositions were verified by amino acid analysis.

Induction and Expansion of Insulin-specific CTLs. Primary CTLs were induced by in vitro stimulation with denatured BI chymotryptic digest. Primary cultures were established by sensitizing 8–10 $\times 10^7$ spleen cells from female C57BL/6 ($H\text{-}2^b$) mice with 4 μM chymotryptic digest of reduced BI per T-25 flask in 10 ml RP-10 media. After a 7-d incubation, the primary cultures were stimulated by mixing the primary effector cells (5×10^6) with 4 μM of the insulin chymotrypsin digest plus irradiated syngeneic feeders (5×10^7 C57BL/6 spleen cells irradiated with 3,000 rad) in 10 ml RP-10 per T-25 flask. Cultures were subsequently maintained in 24-well plates (#2646; Costar Data Packaging, Cambridge, MA) by weekly stimulation of $4–5 \times 10^5$ effector cells/well with a denatured insulin chymotryptic digest (4 μM) plus 5×10^6 irradiated syngeneic feeder cells in RP-10 media supplemented with 5% rat Con A SN and 50 mM α-methyl mannoside.

Analysis of Lytic Activity by ^{51}Cr-release Assay. The lytic activity of CTL cultures and clones was assayed in a 3-h ^{51}Cr-release assay. Briefly, this is performed as follows. Target cells are incubated in 100 μl vol RP-10 media plus 200 μCi ^{51}Cr per 10^6 cells for 60 min at 37°C followed by one wash in PBS. Target cells are resuspended in 10 mls RP-10 media and incubated an additional 30 min at 37°C. After a final wash, target cells are resuspended to 2×10^5 cells/ml in RP-10 media, and 50 μl/well is added to 96-well round-bottomed microtiter plates. Insulin digests and peptides, to be tested as potential antigens, are resuspended to an initial concentration of 8–16 μM in RPMI 1640 media plus 2% BSA without serum, and a 50 μl vol is added to each well containing ^{51}Cr-labeled target cells. Target cells and antigens are then incubated for 15 min at 37°C. Effector CTL populations and clones are resuspended appropriately to yield the final desired effector/target ratios in RP-10 media, and 100 μl is added to each well as appropriate. Since the final vol in each well is 200 μl, the final antigen concentrations tested in this assay are 2–4 μM. After incubation at 37°C in 7% CO_2 for 3 h, the assay plates are centrifuged for 7 min. One-half the vol (100 μl) is collected from each well and transferred to 6 × 50-mm tubes for determination of total ^{51}Cr counts on an Clinigamma counter (model 1272; LKB Instruments, Turku, Finland). Background radioactivity is determined by collecting supernatants from wells in which RP-10 media was used in place of effector CTLs. Maximum count determinations are obtained from wells to which 100 μl of Triton X-100 was added in place of effector CTLs. The determination of specific lysis for these samples is calculated according to the formula: % specific lysis = 100 × (experimental release − background release/maximum release − background release).

Peptide Competition Assay. Truncated peptides, which do not target lysis by insulin-specific CTLs, were examined in a peptide competition assay to determine if their addition in a ^{51}Cr-release assay inhibits CTL-mediated lysis directed against the target antigenic peptide. Briefly, this procedure is conducted as follows: A constant excess amount of truncated peptide (10 μM) is added to ^{51}Cr-labeled target cells. Then titrated quantities of the target peptide, ranging from 4 μM–5 nM, are added to appropriate wells. After a 15-min incubation of peptides and target cells, CTL effector cells are added to give the appropriate E/T ratios, and the assay is allowed to proceed by incubation at 37°C for 3 h. Percent specific lysis is calculated as described above for determination of lytic activity.

Results

Induction and Initial Characterization of Insulin Peptide-specific Cytotoxic T Lymphocytes. A modification of the approach

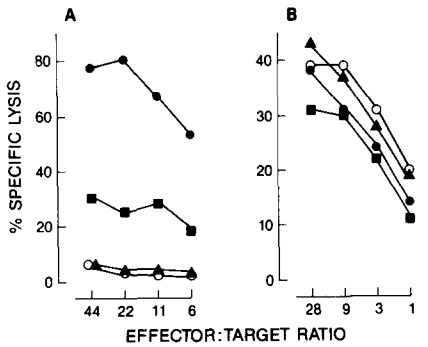

Figure 1. Reactivity of bulk population of C57BL/6-derived CTLs induced by in vitro sensitization with a chymotryptic digest of bovine insulin. (*A*) Response directed against syngeneic EL4 (H-2^b) targets in the presence of 4 μM (●) chymotryptic digest of denatured bovine insulin, (■) chymotryptic digest of undenatured insulin, (▲) native insulin, and (O) no antigen. (*B*) Reactivity against allogeneic P815 (H-2^d) targets with antigens as indicated above.

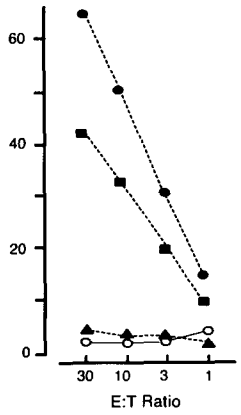

Figure 2. Responses of bulk population of B6-derived CTLs against EL4 targets in the presence of (● – – – ●) 4 μM denatured insulin chymotryptic digest; (■ – – – ■) 4 μM insulin B chain digest; (▲ – – – ▲) 4 μM denatured A chain digest; and (O——O) no antigen.

described by Carbone et al. (19), to induce peptide-specific CTLs by in vitro peptide stimulation with an enzymatic digest of purified protein, was employed to produce insulin peptide-specific CTLs. Spleen cells from C57BL/6 (H-2^b) female mice were used to establish primary (1°) in vitro cultures by stimulation with a complete 5% (wt/wt) chymotryptic digest of denatured BI. Briefly, 8–10 × 10^7 spleen cells were incubated in 10 ml RP-10 with a 4 μM concentration of the chymotryptic denatured BI digest. After 5 d in culture, the primary CTL effector cells frequently demonstrated only minimal antigen-specific reactivity (data not shown). After several passages of in vitro restimulation all cultures consistently exhibited a significant antigen-specific CTL response against syngeneic H-2^b target cells. The results shown in Fig. 1 *A* demonstrate that C57BL/6-derived CTLs lyse syngeneic EL4 (H-2^b) target cells in the presence of a 4-μM concentration of denatured BI chymotryptic digest, but not in the presence of the same concentration of native BI.

A response of much lower magnitude is observed in the presence of undenatured BI chymotryptic digest, suggesting that the chymotryptic fragment generated by digestion of denatured insulin may include one of the cysteine residues involved in the interchain disulfide bonds between the A and B chains. Alternatively, it is possible that the single intrachain disulfide bond between residues A6 and A11 in the A chain must be disrupted to achieve maximum CTL responsiveness of a chymotryptic fragment from the A chain. To distinguish between these possibilities, we tested chymotryptic digests of purified insulin B chain and purified, reduced A chain as antigens for targeting the lytic response of the insulin peptide CTLs against EL4 target cells. The results shown in Fig. 2 demonstrate that the B6-derived CTL response is directed against a fragment(s) from the insulin B chain chymotryptic digest. Furthermore, the absence of any CTL activity against the chymotryptic digest of reduced insulin A chain indicates that the CTL response is specific only for a B chain fragment(s).

Although H-2^b-restricted, insulin digest-specific CTLs respond in an antigen-specific manner on syngeneic target cells, they also exhibit an alloreactive response on allogeneic P815 (H-2^d) target cells, demonstrating significant lysis in the absence of insulin chymotryptic peptides which is unaltered by the addition of specific antigen (Fig. 1 *B*). All clones derived from this CTL population also exhibit this dual reactivity (data not shown), indicating that each CTL in this population possesses both antigen-specific and alloreactive specificities. Dual-reactive T cells, which recognize allogeneic MHC in the absence of foreign antigen, in addition to syngeneic MHC plus foreign antigen, have been previously described for both class II–restricted T cells (24, 25) and class I–restricted CTLs (26, 27). In addition, "promiscuous CTLs," which recognize foreign antigen in the context of a variety of class I MHC alleles have been isolated (19, 28, 29). The insulin peptide-specific CTLs described here are unresponsive against the H-2^k-expressing target cells, CBA.D1 and LMTK$^-$, even in the presence of antigen (data not shown). Furthermore, class I MHC-restricted peptide recognition by the insulin-specific CTLs occurs only in the context of syngeneic H-2^b target cells. Thus the functional reactivity of these CTLs is similar to other dual-reactive, antigen-specific T cells that, in addition to their primary specificity, exhibit an additional alloreactivity, which in this case is directed against target cells expressing allogeneic H-2^d class I molecules. At the present time, the precise nature of the H-2^d class I specificity of this alloreactive response is undetermined.

Identification of Insulin B Chain Peptide as the Target Antigen. To determine the primary sequence of the insulin B chain chymotryptic fragment which constitutes the target antigen for these CTLs, we performed a preparative HPLC analysis on 10 mg of the insulin B chain chymotryptic digest. The HPLC profile shown in Fig. 3 *A* reveals that 11 major discrete peaks, presumably representing individual chymotryptic fragments, could be separated in this digest preparation. Each peak was recovered as an individual fraction for use as a potential antigen for targeting the CTL-mediated lysis against syngeneic EL4 target cells. Fractions were resuspended to the same molar concentration found in the complete digest preparation for analysis of antigenic activity. Thus, all fractions were tested at a final 4 μM equivalent con-

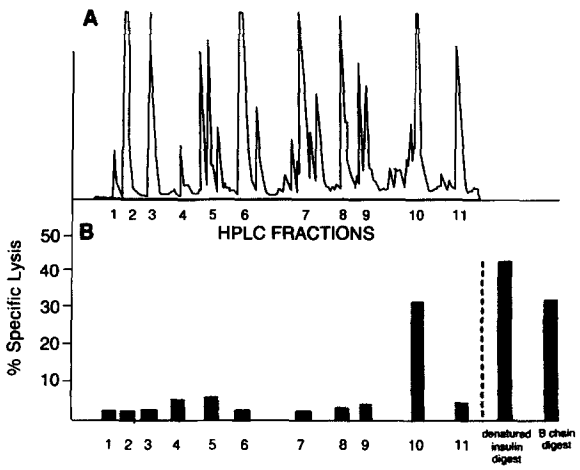

Figure 3. (*A*) Preparative HPLC profile of purified insulin B chain chymotryptic digest, showing 11 fractions collected individually for analysis for antigenic reactivity. (*B*) Response of CTL clone 10.5.4 (30:1 E/T ratio) against 4 μM equivalent concentration of the 11 HPLC fractions, and against denatured insulin digest and insulin B chain digest on EL4 targets.

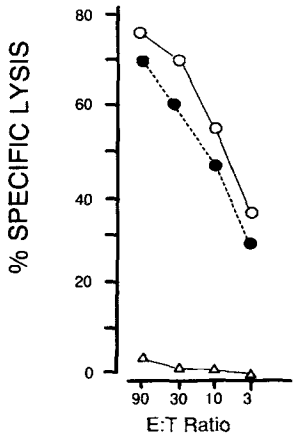

Figure 4. Clone 10.5.4 response against 4 μM (● – – – ●) denatured insulin digest; (O——O) insulin B chain peptide, p7–15; and (Δ——Δ) no antigen.

centration in a 3-h ^{51}Cr-release assay. The results shown in Fig. 3 *B* reveal the lytic response for one of several representative cloned CTL lines isolated from these B6-derived CTLs, designated clone 10.5.4, and depict the antigenic activity present in each fraction represented in Fig. 3 *A*, as well as the corresponding activity in the presence of chymotryptic digests of denatured insulin and insulin B chain. It is clear from these results that all of the antigenic reactivity in the insulin B chain chymotryptic digest resides in fraction 10. The results from amino acid composition analysis of fraction 10 were compared with the primary sequence of the insulin B chain. Accordingly, a single chymotryptic fragment, corresponding to the B chain peptide p7–15, was identified in this fraction. The primary sequence of this peptide is: Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu, derived by chymotryptic cleavage on the carboxyl-side of the leucine residues at positions 6 and 15 of the B chain. To confirm that this nine-

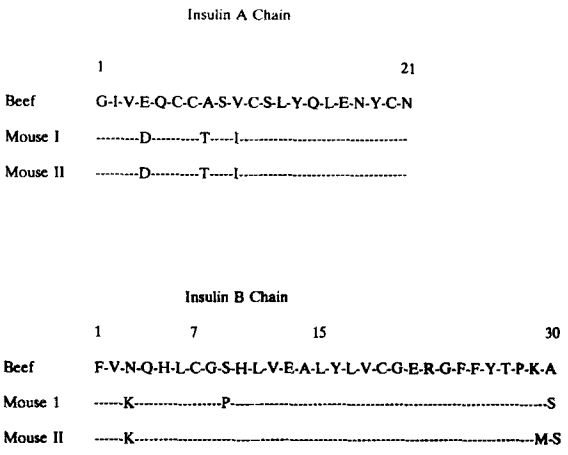

Figure 5. Comparison of primary sequences for A and B chains from bovine insulin, and murine I and murine II insulin.

residue B chain peptide constitutes the target antigen, a peptide corresponding to the above sequence was synthesized and tested for its reactivity with clone 10.5.4 on EL4 target cells. As shown in Fig. 4, the response to this synthetic peptide is similar to that seen against the chymotryptic digest of the denatured insulin preparation, thus confirming the insulin B chain peptide, p7–15, as the target antigen.

In the mouse, insulin occurs in two related forms, designated mouse insulin I and II (30). The primary sequences of both forms are shown in Fig. 5 in comparison with BI which was used as the antigen in this study. Of the 51 residues which comprise both the A and B chains, both forms of murine insulin differ from the bovine sequence by only six residues, and mouse insulin I differs from mouse insulin II in two residues, a Pro to Ser change at position 9, and a Lys to Met change at position 29 in the B chain. Thus the insulin B chain p7–15 primary sequence in mouse insulin II is identical to that of BI, and the mouse insulin I form differs by a single Ser to Pro substitution at position 9. Since all individuals of the murine species express both insulin I and II, both forms are "recognized" by the immune system as self-proteins. Thus the BI B chain p7–15 chymotryptic fragment, which serves as the target antigen for these B6-derived CTLs, is an autologous peptide, at least with respect to that found in the mouse insulin II form.

CTL Recognition of Insulin B Chain p7–15 Is H-2K^b Restricted. To further characterize the B6-derived CTLs which respond to the insulin B chain peptide p7–15 on syngeneic target cells, we sought to identify the specific H-2^b molecule which serves as the restriction element for this response. Using L cell fibroblast lines transfected with class I H-2^b genes as target cells, we assessed whether the p7–15-specific CTLs could respond to the insulin chymotryptic digest and insulin B chain p7–15 in the context of the D^b and K^b class I molecules. As shown in Fig. 6, CTL recognition of this insulin peptide by the B6-derived CTL clone 10.5.4 is clearly restricted to the H-2K^b class I molecule. This is demonstrated by the strong lytic response against transfected L-K^b

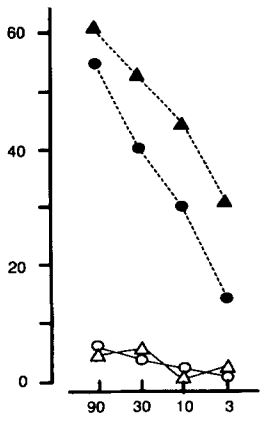

Figure 6. Response of clone 10.5.4 against LK^b transfected targets in the presence of 4 μM (● – – – ●)denatured insulin digest and (▲ – – – ▲) insulin B chain peptide, p7–15; and against LD^b transfected targets in the presence of (O——O) denatured insulin digest and (Δ——Δ) insulin B chain peptide, p7–15.

target cells in the presence of both denatured insulin digest and the synthetic p7–15 antigens and by the lack of such response on transfected L-D^b target cells. The bulk CTL effectors, from which clone 10.5.4 is derived, exhibit the same class I MHC restriction in the presence of this peptide (data not shown).

Determination of the Minimal Antigenic Peptide Derived from Insulin B Chain, p7–15. To identify the minimal peptide necessary for CTL recognition, we synthesized various truncated peptides derived from the insulin B chain chymotryptic fragment to determine the influence of individual residues in this sequence to H-2K^b-restricted CTL recognition. The results of this analysis are shown in Table 1. They reveal that removal of the amino acid residues Cys^7, Gly^8, and Ser^9, from the amino-end of p7–15 has no effect on CTL recognition since peptides p8–15, p9–15, and p10–15 are undiminished in their antigenicity with respect to recognition by these B6-derived CTLs. The effect of removing the carboxyl-terminal Leu residue at position 15, however, results in abrogation of CTL recognition as indicated by the lack of CTL-mediated lysis in the presence of either p9–14 or p10–14. Thus, from this analysis, we conclude that the minimal antigenic sequence

Table 1. *Determination of Minimum Antigenic Peptide Reactivity for Clone B6.BI-10.5.4*

Peptide*	Sequence	CTL reactivity‡
p7–15	C-G-S-H-L-V-E-A-L	58
p8–15	G-S-H-L-V-E-A-L	55
p9–15	S-H-L-V-E-A-L	54
p10–15	H-L-V-E-A-L	55
p11–15	L-V-E-A-L	3
p9–14	S-H-L-V-E-A	2
p10–14	H-L-V-E-A	3

* All peptides were tested for antigenic activity at a final concentration of 2 μM in a 3-h ^{51}Cr-release assay with ^{51}Cr-labeled EL4 target cells.
‡ Clone B6.BI-10.5.4 cells were used at an E/T ratio of 30:1.

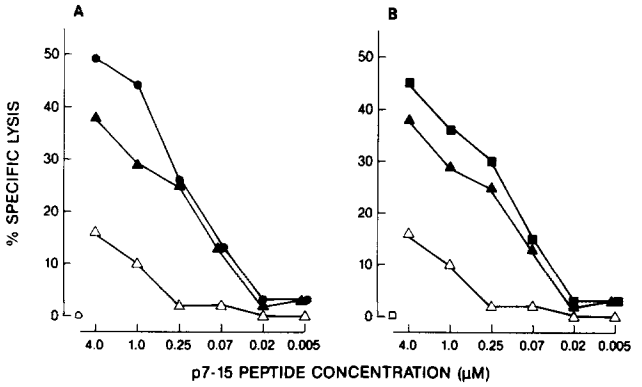

Figure 7. Peptide competition on EL4 targets with clone 10.5.4 effectors: in both *A* and *B* ▲ denotes the response against titrated concentrations from 4 μM to 5 nM of insulin B chain peptide p7–15; and Δ, the response against p7–15 in the presence of 10 μM concentration of VSV N protein peptide p49–59. Clone 10.5.4 cells were used at a 10:1 E/T ratio. (*A*) (●), Titration of p7–15 response in the presence of 10 μM p10–14 truncated insulin B chain peptide; O, lytic response to 10 μM concentration of p10–14; (*B*) (■), Titration of p7–15 response in the presence of 10 μM p11–15 truncated insulin B chain peptide; □, lytic response to 10 μM concentration of p11–15.

of the chymotryptic fragment, p7–15, is His-Leu-Val-Glu-Ala-Leu, which comprises the insulin B chain peptide, p10–15. This core antigenic peptide does not include the Pro→Ser sequence difference between mouse insulin I and BI at residue 9 (see Fig. 5). Thus, the sequence p10–15 of the target antigen is an autologous peptide in both forms of mouse insulin, and the B6-derived CTLs that are responsive to this peptide may be considered autoreactive CTLs.

To evaluate the contribution of individual residues in this peptide to MHC/peptide binding or TCR/peptide contact, we initiated peptide competition studies using an excess amount (10 μM) of truncated peptides, which were not antigenic with respect to CTL lysis of EL4 target cells, added together with titrated quantities of p7–15 (from 4 μM–5 nM). As a control for peptide/MHC binding, we used the vesicular stomatitis virus (VSV) nuclear (N) protein peptide, p49–59, which has been shown previously to be a target antigen for lysis by K^b-restricted, VSV-specific CTLs (31). Using the antigenic peptide, p7–15, as the target antigen for CTL-mediated lysis we tested the inactive truncated peptides, p10–14 (Fig. 7 *A*) and p11–15 (Fig. 7 *B*), for competition of p7–15 peptide binding to L+K^b target cells. The results shown in Fig. 7 demonstrate that the addition of 10 μM VSV N protein p49–59 peptide, which fails to target lysis by the insulin peptide-specific CTLs, inhibits the CTL response against insulin B chain p7–15. This suggests that VSV N protein p49–59 binds to the class I H-2K^b molecule in a manner such that it inhibits binding of the insulin B chain p7–15. However, addition of excess insulin B chain truncated peptides p10–14 and p11–15, derived by the removal of Leu^{15} and His^{10} residues respectively, failed to inhibit the K^b-restricted CTL lysis of EL4 target cells in the presence of antigenic peptide p7–15. Indeed, particularly in the presence of the p10–14 peptide, there appears to be some increase in

activity by clone 10.5.4 with higher concentrations of the p7–15 peptide. These findings indicate that removal of either Leu^{15} or His^{10} abrogates peptide binding to the K^b molecule. Currently we are evaluating each of the residues in the minimal antigenic core peptide, p10–15, with respect to their classification as H-2K^b-binding residues or TCR-interacting residues.

Discussion

To examine the antigenicity of insulin with respect to a class I H-2-restricted CTL response, we have induced insulin peptide-specific CTLs from C57BL/6 (H-2^b) mouse spleen cells by in vitro stimulation with a chymotryptic digest of BI. The $CD8^+$ CTLs induced in this manner, as described in this study, are specific for an autologous peptide derived from the B chain of the insulin molecule. In contrast to the results described in this paper, peptides from both the A and the B chain of insulin have been shown to be antigenic in the class II–restricted Th response to insulin. Furthermore, Delovitch and co-workers (5, 32, 33) have demonstrated that major epitopes for Th recognition of insulin are represented by the branched peptides, A1–14/B7–15, A1–14/B7–16, and A1–14/B7–26, which are comprised of peptide fragments from both the A and B chains of insulin connected by an interchain disulfide bond between residues A7 and B7. In another report (8) it was demonstrated that the Th response to an insulin A chain–derived peptide could be inhibited by an insulin B chain peptide, p17–26. Thus far we have failed to identify class I H-2-restricted T cells, specific for either A chain or branched peptides derived from both the A and B chains of insulin, from cultures of C57BL/6 spleen cells stimulated in vitro with chymotryptic digests of either reduced insulin or native, oxidized insulin. This may suggest that, under the conditions used in this study, the insulin B chain p7–15 chymotryptic fragment may be immunodominant, thereby preventing our detection of class I–restricted CTLs specific for branched peptides or peptides derived from the A chain. Indeed, CTLs specific for this same p7–15 insulin B chain fragment have emerged from several cultures induced in this manner.

However, the antigenic peptide identified in this study is similar to a Th epitope of insulin B chain in C57BL/6 mice, p7–16, reported by Jensen (9). Recognition of this peptide by class II–restricted Th cells is dependent on the Cys^7 residue, as modification of this amino acid interferes with the antigenicity of the peptide. Our determination, in this study, of insulin B chain–derived p10–15 as the minimal peptide necessary for targeting the antigen-specific lytic activity of these B6-derived CTLs, however, clearly rules out any influence on CTL recognition by not only the Cys^7 residue, but also by the Gly^8 and Ser^9 residues of the B chain. Thus, to our knowledge, the CTL reactivity described in this study is unique with respect to T cell recognition of insulin peptides, and furthermore, these results represent the first reported evidence of a class I MHC-restricted, insulin peptide-specific CTL response.

Although the core antigenic peptide has been identified as the hexamer, p10–15, it should be noted that the chymotryptic fragment responsible for induction of these insulin peptide-specific CTLs is the nonamer peptide, p7–15. In agreement with recent observations by several groups (31, 34–36), this peptide conforms to the proposed length of "naturally processed" peptides presented in the class I MHC presentation pathway as being either nonamers or octamers. Thus, although the Cys^7, Gly^8, and Ser^9 residues are not required for K^b-restricted CTL recognition, they may be important for either appropriate processing or presentation in the class I pathway. Also worth noting is the predominantly hydrophobic character of this K^b-presented peptide, which similarly is characteristic of the class II–presented insulin peptides (37). As suggested by Lu et al. (38), the presence of recurrent hydrophobic residues in this CTL-recognized insulin peptide may be important to enable it to bind more efficiently to class I H-2K^b molecules, thereby increasing the likelihood that it will be expressed on the cell surface in a manner appropriate for T cell recognition. The occurrence of hydrophobic residues at anchor positions two residues apart has also been identified in naturally processed peptides eluted from the K^b molecule (36).

The specificity of these H-2K^b-restricted CTLs for the autologous mouse insulin II B chain peptide, p7–15, demonstrates that potentially autoreactive, insulin peptide-specific T cell receptors are expressed in the mature peripheral $CD8^+$ T lymphocyte repertoire of the C57BL/6 mouse. That CTLs with this specificity can exist in the periphery, without any obvious detrimental effects to the insulin-producing pancreatic β cells, raises questions concerning their potential in vivo relevance. In this regard it should be considered that, in the present study, induction of these autoreactive CTLs may be dependent upon a Th epitope present in bovine insulin, but perhaps absent from murine insulin, which is exposed by in vitro digestion with α-chymotrypsin. It is conceivable that activation of an insulin-responsive $CD4^+$ Th population could provide a source for IL-2, which might be necessary for the induction and/or expansion of these potentially autoreactive $CD8^+$ CTLs. Indeed, a similar mechanism has been documented (39) for the expansion of $CD8^+$ tumor-specific CTLs by immunizing mice with cells from IL-2-secreting lines. Thus the absence of this Th epitope in murine insulin would fail to activate these IL-2-producing $CD4^+$ Th cells, thereby precluding the expansion of this $CD8^+$-autoreactive CTL population. If this is the primary means of regulating this potentially autoreactive CTL population, then perhaps the induction and expansion of this population in vitro, as described in the present study, may overcome this requirement for $CD4^+$ Th cells.

Alternatively this potentially autoreactive CTL population may remain dormant in normal C57BL/6 mice due to the absence or low level of antigenic stimulation. Normal pancreatic β cell metabolism results in the synthesis of proinsulin followed by cleavage of the C peptide to yield the mature form of insulin. This hormone is then secreted in its native, intact form. A low level of insulin processing may occur through normal intracellular degradation in the pancreatic β cells, thereby perhaps helping to maintain this dor-

mant insulin peptide-specific CTL population in the periphery. We are presently investigating in diabetic NOD mice whether such an insulin-derived peptide, perhaps generated by abnormal metabolism in the diabetic pancreas, may serve as a target antigen for the activation of autoimmune CTL effectors in IDDM.

We thank Mr. Qiang Liu for expert technical assistance in the preparation, isolation, and purification of native and synthetic insulin peptides. We also thank Dr. S. G. Nathenson for providing the VSV N protein peptide, p49–59.

This work was supported by National Institutes of Health grants AI-27917 to J. M. Sheil, and GM-31841 to Y. Paterson, who is also supported by an American Cancer Society Faculty Research Award.

Address correspondence to Dr. James M. Sheil, Department of Microbiology and Immunology, West Virginia Health Sciences Center, Morgantown, WV 26506; or to Dr. Yvonne Paterson, Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104.

Received for publication 30 August 1991 and in revised form 21 October 1991.

Note added in proof: The mouse insulin I–derived B chain p7-15 peptide, which contains a Pro^9 residue in place of Ser^9, also targets lysis by clone 10.5.4 as efficiently as the bovine and mouse insulin II B chain p7-15 peptide.

References

1. Barcinski, M.A., and A.S. Rosenthal. 1977. Immune response gene control of determinant selection. I. Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. *J. Exp. Med.* 145:726.
2. Ishii, N., J. Klein, and Z.A. Nagy. 1983. Different repertoires of mouse T cells for bovine insulin presented by syngeneic and allogeneic cells. *Eur. J. Immunol.* 13:658.
3. Spaeth, E., and E. Rude. 1983. Epitope specificity and Ia restriction of T cell responses to insulin in a system of complementing Ir genes: analysis with primed lymph node T cells and a long-term cultured T cell line. *Eur. J. Immunol.* 13:756.
4. Glimcher, L.H., J.A. Schroer, C. Chan, and E.M. Shevach. 1983. Fine specificity of cloned insulin-specific T cell hybridomas: evidence supporting a role for tertiary conformation. *J. Immunol.* 131:2268.
5. Naquet, P., J. Ellis, B. Singh, R.S. Hodges, and T.L. Delovitch. 1987. Processing and presentation of insulin. I. Analysis of immunogenic peptides and processing requirements for insulin A loop-specific T cells. *J. Immunol.* 139:3955.
6. Thomas, J.W., W. Danho, E. Bullesbach, J. Fohles, and A.S. Rosenthal. 1981. Immune response gene control of determinant selection. III. Polypeptide fragments of insulin are differentially recognized by T but not by B cells in insulin immune guinea pigs. *J. Immunol.* 126:1095.
7. Huber, B.T., and P.S. Hochman. 1984. B cell activation potential of insulin-reactive T cells in $H\text{-}2^b$ mice. *Eur. J. Immunol.* 14:1106.
8. Miller, G.G., J.F. Hoy, and J.W. Thomas. 1989. Insulin B chain functions as effective competitor of antigen presentation via peptide homologies present in the thymus. *J. Exp. Med.* 169:2251.
9. Jensen, P. 1990. Immunogenicity of B chain in insulin responder and nonresponder mice. *Cell. Immunol.* 130:129.
10. Todd, J.A., J.I. Bell, and H.O. McDevitt. 1988. A molecular basis for genetic susceptibility to insulin-dependent diabetes mellitus. *Trends Genet.* 4:129.
11. Wang, Y., L. Hao, R.G. Gill, and K.J. Lafferty. 1987. Autoimmune diabetes in NOD mouse is L3T4 T-lymphocyte dependent. *Diabetes.* 36:535.
12. Charlton, B., and T.E. Mandel. 1988. Progression from insulitis to β-cell destruction in NOD mouse requires $L3T4^+$ T-lymphocytes. *Diabetes.* 37:1108.
13. Boitard, C.H., R. Yasunami, M. Dardenne, and J.F. Bach. 1989. T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J. Exp. Med.* 169:1669.
14. Haskins, K., M. Portas, B. Bergman, K. Lafferty, and B. Bradley. 1989. Pancreatic islet-specific T-cell clones from nonobese diabetic mice. *Proc. Natl. Acad. Sci. USA.* 86:8000.
15. Charlton, B., A. Bacelj, and T.E. Mandel. 1988. Administration of silica particles or anti-Lyt2 antibody prevents β-cell destruction in NOD mice given cyclophosphamide. *Diabetes.* 37:930.
16. Nagata, M., K. Yokono, M. Hayakawa, N. Hatamori, W. Ogawa, K. Yonezawa, K. Shii, and S. Baba. 1989. Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice. *J. Immunol.* 143:1155.
17. Bellgrau, D., and A.-C. LaGarde. 1990. Cytotoxic T-cell precursors with low-level CD8 in the diabetes-prone Biobreeding rat: Implications for generation of an autoimmune T-cell repertoire. *Proc. Natl. Acad. Sci. USA.* 87:313.
18. Parham, P. 1988. Intolerable secretion in tolerant transgenic mice. *Nature (Lond.).* 333:500.
19. Carbone, F.R., M.W. Moore, J.M. Sheil, and M.J. Bevan. 1988. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. *J. Exp. Med.* 167:1767.
20. Carbone, F.R., B.S. Fox, R.H. Schwartz, and Y. Paterson. 1987. The use of hydrophobic α-helix-defined peptides in delineating the T cell determinant for pigeon cytochrome c. *J. Immunol.* 138:1838.
21. Bhayani, H., F.R. Carbone, and Y. Paterson. 1988. The acti-

vation of pigeon cytochrome c-specific T cell hybridomas by antigenic peptides is influenced by non-native sequences at the amino terminus of the determinant. *J. Immunol.* 141:377.
22. Barany, G., and R.B. Merrifield. 1980. Solid-phase peptide synthesis. *In* The Peptides: Analysis, Synthesis, and Biology. Vol. 2. S. Udenfriend, and J. Meienhofer, editors. Academic Press, New York. 1–284.
23. Tam, J.M., and R.B. Merrifield. 1987. Strong acid deprotection of synthetic peptides: mechanisms and methods. *In* The Peptides: Analysis, Synthesis, and Biology, Vol. 9. S. Udenfriend, and J. Meienhofer, editors. Academic Press, New York. NY. 185–248.
24. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C.A. Janeway, Jr. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med.* 158:836.
25. Fink, P.J., L.A. Matis, D.L. McElligot, M.A. Bookman, and S.M. Hedrick. 1986. Correlations between T-cell specificity and the structure of the antigen receptor. *Nature (Lond.).* 321:219.
26. Sheil, J.M., M.J. Bevan, and L. Lefrancois. 1987. Characterization of dual-reactive H-2K^b-restricted anti-vesicular stomatitis virus and alloreactive cytotoxic T cells. *J. Immunol.* 138:3654.
27. Bevan, M.J. 1977. Killer cells reactive to altered-self antigens can also be alloreactive. *Proc. Natl. Acad. Sci. USA.* 74:2094.
28. Frelinger, J.A., F.M. Gotch, H. Zweerink, E. Wain, and A.J. McMichael. 1990. Evidence of widespread binding of HLA class I molecules to peptides. *J. Exp. Med.* 172:827.
29. Sherman, L.A., and A.M. Lara. 1989. Unrestricted recognition of a nonapeptide antigen by CD8$^+$ cytolytic T lymphocytes. *J. Immunol.* 143:3444.
30. Talmon, J., G. Ranghino, A. Yonath, and I.R. Cohen. 1983. Structural analysis of insulin determinants seen by T cells directed by H-2 genes. *Immunogenetics.* 18:79.
31. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:213.
32. Naquet, P., J. Ellis, D. Tibensky, A. Kenshole, B. Singh, R. Hodges, and T.L. Delovitch. 1988. T cell autoreactivity to insulin in diabetic and related non-diabetic individuals. *J. Immunol.* 140:2569.
33. Semple, J.W., J. Ellis, and T.L. Delovitch. 1989. Processing and presentation of insulin. II. Evidence for intracellular, plasma membrane-associated and extracellular degradation of human insulin by antigen-presenting B cells. *J. Immunol.* 142:4184.
34. Schumacher, T.N.M., M.L.H. De Bruijn, L.N. Vernie, W.M. Kast, C.J.M. Melief, J.J. Neefjes, and H.L. Ploegh. 1991. Peptide selection by MHC class I molecules. *Nature (Lond.).* 350:703.
35. Rotzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
36. Falk, K., O. Rotzscke, and H.-G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.).* 348:248.
37. Delovitch, T.L., A.H. Lazarus, M.L. Phillips, and J.W. Semple. 1989. Antigen binding and processing by B-cell antigen presenting cells: influence on T- and B-cell activation. *Cold Spring Harbor Symp. Quant. Biol.* 54:333.
38. Lu, S., V.E. Reyes, R.A. Lew, J. Anderson, J. Mole, R.E. Humphreys, and T. Ciardelli. 1990. Role of recurrent hydrophobic residues in catalysis of helix formation by T cell-presented peptides in the presence of lipid vesicles. *J. Immunol.* 145:899.
39. Fearon, E.R., D.M. Pardoll, T. Itaya, P. Golumbek, H.I. Levitsky, J.W. Simons, H. Karasuyama, B. Vogelstein, and P. Frost. 1990. T helper function in the generation of an antitumor response. *Cell.* 60:397.