Transporters Associated with Antigen Processing (TAP)-independent Presentation of Soluble Insulin to α/β T Cells by the Class Ib Gene Product, Qa-1^b

By S. Mark Tompkins,* Jennifer R. Kraft,* Chinh T. Dao,* Mark J. Soloski,‡ and Peter E. Jensen*

*From the *Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322; and the ‡Division of Molecular and Clinical Rheumatology, Department of Medicine, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205*

Summary

T cell hybridomas isolated from nonresponder H-2^b mice immunized with pork insulin were stimulated by insulin in the presence of major histocompatibility complex (MHC)-unmatched antigen presenting cells. The restriction element used by these CD4$^-$ T cells was mapped to an oligomorphic MHC class Ib protein encoded in the T region and identified as Qa-1^b using transfectants. The antigenic determinant was localized to the insulin B chain, and experiments with truncated peptides suggested that it is unexpectedly long, comprising most or all of the 30 amino acid B chain. The antigen processing pathway used to present insulin to the Qa-1^b–restricted T cells does not require transporters associated with antigen processing (TAP), and it is inhibited by chloroquine. A wide variety of cell lines from different tissues efficiently present soluble insulin to Qa-1^b–restricted T cells, and insulin presentation is not enhanced by phagocytic stimuli. Our results demonstrate that Qa-1^b can function to present exogenous protein to T cells in a manner similar to MHC class II molecules. Therefore, this class Ib protein may have access to a novel antigen processing pathway that is not available to class Ia molecules.

Key words: antigen processing • major histocompatibility proteins • class Ib molecules • T cells • insulin

The family of MHC class I–related molecules in mice includes the "classical" (class Ia) proteins H-2K, D, and L, "nonclassical" (class Ib) molecules encoded in the Q, T, and M regions, and β_2-microglobulin (β_2m)[1]-associated molecules such as CD1 that are encoded by genes not linked to the MHC (1–3). In most species, the class Ib genes significantly outnumber the class Ia genes but the proportion of class Ib pseudogenes is relatively high. Compared with class Ia genes, class Ib genes are relatively nonpolymorphic, with few alleles and relatively small differences between alleles. The tissue distribution of class Ib gene products is heterogeneous, and expression levels are generally lower than those observed for class Ia proteins. Although the function of class Ib molecules remains unknown, it is likely that different molecules may have different functions, and there is a growing body of evidence that certain nonclassical class I proteins have specialized antigen presentation function. For example, H-2M3 has a specialized capacity to present *N*-formylated peptides which, regardless of sequence, bind poorly to class Ia molecules (4). This protein may have evolved to play a special role in the immune response to bacteria (5). Human CD1b is selectively targeted to endosomal compartments (6), where it can bind and present bacterial lipid antigens to TCR α/β T cells (7, 8).

The T region–encoded Qa-1 molecule, first identified serologically (9), has long been considered as a transplantation antigen that can be recognized by alloreactive CTLs (10–13). Qa-1 is expressed in tissues with a distribution similar to class Ia proteins but cell-surface density is relatively low. Four Qa-1 alleles have been characterized by serological and CTL assays, and the genes encoding two of these, Qa-1^b (14, 15) and Qa-1^a (16), have been cloned. A large fraction of Qa-1^b–reactive TCR α/β CTLs have been shown to recognize a nonameric peptide, Qdm (Qa-1 determinant modifier), derived from the signal sequence of

[1]*Abbreviations used in this paper:* β_2m, β_2-microglobulin; BFA, brefeldin A; ER, endoplasmic reticulum; Ii, class II invariant chain; IR, insulin receptor; Qdm, Qa-1 determinant modifier; SEB, staphylococcal enterotoxin B; TAP, transporters associated with antigen processing.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/09/961/11 $2.00
Volume 188, Number 5, September 7, 1998 961–971
http://www.jem.org

class Ia H-2D and L molecules (17, 18). Peptide extraction studies suggest that the peptides bound to Qa-1^b molecules in activated T cells and transfected cells have limited heterogeneity, and that Qdm is the dominant peptide associated with this protein (18–20). Additional studies suggest that other antigens may also be presented by Qa-1^b. Vidovic et al. (21) reported the isolation of a Qa-1^b–restricted TCR γ/δ T cell hybridoma that recognizes the amino acid polymer poly($Glu^{50}Tyr^{50}$). Imani and Soloski (22) demonstrated that cell surface Qa-1^b expression is selectively upregulated after heat shock. A tryptic digest of *Mycobacterium bovis* 65-kD heat shock protein was observed to stabilize cell surface Qa-1^b molecules, suggesting that this protein may be involved in presenting heat shock protein–derived peptides to the immune system. Jiang et al. (23) reported that Qa-1^b–restricted CTLs are involved in peripheral deletion of TCR V$\beta8^+$ $CD4^+$ T cells after injection with staphylococcal enterotoxin B (SEB) superantigen. These CTLs killed TCR V$\beta8^+$ but not V$\beta8^-$ T cells, suggesting that they recognize an endogenous TCR Vβ-derived peptide(s) presented by Qa-1 (23). Bouwer et al. (24) demonstrated recently that Qa-1^b–restricted CTLs are induced after infection with the intracellular pathogen, *Listeria monocytogenes*. These CTLs specifically lyse Qa-1^b–expressing, *L. monocytogenes*–infected cell lines, indicating that they directly recognize a bacterial antigen presented by Qa-1. Thus, it is clear that Qa-1 can function as an antigen-presenting molecule in a variety of situations, but generalizations concerning its role in the immune system are difficult to make.

In this study, we describe T cell hybridomas isolated from nonresponder H-2^b mice immunized with pork insulin that recognize an insulin B chain determinant presented by Qa-1^b. The antigen processing pathway used to present insulin to these T cells appears to be distinct from the unconventional pathway(s) described previously for the presentation of exogenous protein antigens to MHC class Ia–restricted T cells.

Materials and Methods

Protein and Peptide Antigens. Beef and pork insulin were purchased from Elanco Animal Health (Indianapolis, IN). The desoctapeptide of beef insulin and sulfonated B chain were generated as described previously (25). Sperm whale myoglobin was purchased from Sigma Chemical Co. (St. Louis, MO). Peptides were synthesized using a peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA) in the Emory University Microchemical Facility or by using F-MOC chemistry with a Symphony multiple peptide synthesizer (Rainin Instrument Co., Inc., Woburn, MA).

Mice. Female C57BL/10SnJ (B10), C57BL/6J (B6), B10.BR/SgSnJ, C3H/HeJ, BALB/cByJ, A/J, A.BY/SnJ, and SJL/J were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II–negative, C2D (A$\beta^{-/-}$) mice were purchased from GenPharm International (Mountain View, CA). C57BL/6J-*$\beta2m^{tm1Unc}$* ($\beta_2m^{-/-}$) breeder pairs were purchased from The Jackson Laboratory, and maintained by the Emory University Animal Facilities in specific pathogen–free conditions. H-2M–deficient, B6,129-*H2-Ma^{tm1Luc}* (H2-$Ma^{-/-}$) mice (26) and C57BL/6J-*$Tap1^{tm1Arp}$* (Tap$1^{-/-}$) mice (27) were the gift of Dr. Luc van Kaer (Vanderbilt University, Nashville, TN). Spleen cells from class II invariant chain (Ii) knockout mice (28) were provided by Dr. Alexander Y. Rudensky (University of Washington, Seattle, WA). B6-Tla^a and A-Tla^b mice were maintained under pathogen-free conditions at Johns Hopkins School of Medicine. Mice were used at 8–11 wk of age.

Cells and Cell Lines. The Qa-1^b–expressing L cell transfectant, L-g37, and vector control, L-V, were generated as described (22). TH2.2 (29) and LB 27.4 (30) are H-$2^{b\times d}$ B hybridomas, whereas A20 is a BALB/c-derived B lymphoma (31) and EL4.CW (32) is an H-2^b T cell lymphoma. The P815 DBA/2–derived mastocytoma (TIB-64) was obtained from the American Type Culture Collection (Rockville, MD). BW1100-129.237 (33) is a TCR $\alpha/\beta^{-/-}$ derivative of BW5147. The C3H-derived melanoma, K1735-M2 (34), was a gift of Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX), and the F10Q clone of B16 melanoma was obtained from Dr. C. Armstrong (Emory University, Atlanta, GA). Dr. D. Accili (National Institutes of Health, Bethesda, MD) provided transformed hepatocyte cell lines generated from insulin receptor-deficient ($IR^{-/-}$) and $IR^{+/+}$ littermates (35). B3Z, a $CD8^+$ T-T hybridoma specific for OVA 257-264 (SIINFEKL) presented by K^b, was provided by Dr. N. Shastri (University of California, Berkeley, CA). Insulin-specific T cell hybridomas (25) were prepared from C57BL/10SnJ mice immunized subcutaneously with 50 μg pork insulin emulsified in CFA. 9–12 d after immunization, lymphocytes were isolated from draining lymph nodes and cultured for 4 d at 10^7 cells/ml with 100–200 μg/ml pork insulin in RPMI 1640 supplemented with 0.5% freshly prepared normal mouse serum, 2 mM glutamine, 50 μM 2-ME, 10 μg/ml gentamicin, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. Viable lymphocytes isolated on a Ficoll gradient were fused at a ratio of 1:2 or 1:3 with T lymphoma cells and selected in RPMI 1640 supplemented with 20% FCS and 2 mM HAT. T cell hybrids were produced using BW5147 (Pb13, Pb1.4) or 3B2-E7 (6C5, B1A4), a CD8-transfected, TCR $\alpha/\beta^{-/-}$ derivative of BW5147 (36) provided by Dr. P. Gottlieb. Hybrids were expanded in medium containing hypoxanthine and thymidine for initial screening. Primary wells containing cells secreting lymphokine after stimulation with pork insulin in the presence of C57BL/10SnJ splenocytes as APCs were subcloned by limiting dilution. Phenotypes were determined by using indirect immunofluorescence and flow cytometry. All hybridomas express TCR α/β but not TCR γ/δ. Pb13 ($CD4^-CD8^-$), 6C5 ($CD4^-CD8^+$), Pb1.4 ($CD4^+CD8^-$), and B1A4 ($CD4^+CD8^+$) are representative of hybridomas isolated from multiple independent fusions.

Cos Cell Transfection. Transiently transfected Cos cells expressing Qa-1^b were generated using Qa-1^b cDNA cloned into the mammalian expression vector pCDNA3.0 (Invitrogen Corp., Carlsbad, CA). On day 0, 3.0×10^5 Cos (37) cells were plated in 5 ml supplemented DMEM/10% FCS, in 6 cm tissue culture dishes. Cultures were aspirated on day 1 and washed twice with DMEM plus 10 mM Hepes. To each dish, 2 ml DMEM plus 10 mM Hepes was added, containing 1 μg sterile cDNA (or vector only), 500 μg DEAE-dextran, and 20 ml 10 mM chloroquine. Petri dishes were incubated for 3 h at 37°C, 7% CO_2. After incubation, plates were aspirated, and 2 ml of 10% DMSO in DMEM plus 10 mM Hepes was added. Petri dishes were incubated 1–2 min, aspirated, and 5 ml DMEM/10% FBS was added. Plates were incubated for 24–48 h before cell removal by trypsinization and use in T cell assays. Qa-1^b expression was measured using indirect immunofluorescence and flow cytometry with a Qa-1^b–specific rabbit antiserum.

Analysis of Qa-1 Expression by Flow Cytometry. Rabbit antisera were generated with a synthetic peptide from the α2 domain of Qa-1^b, and the IgG fraction was purified using protein A–Sepharose affinity chromatography. Cells (10^6) were incubated with Qa-1^b–specific or control rabbit IgG (10–20 μg/ml) in 50 μl PBS containing 1% FCS for 30–45 min, washed, and further incubated with FITC-labeled goat anti–rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). Fluorescence was analyzed using a FACScan® (Becton Dickinson, San Jose, CA).

Culture Conditions and Lymphokine Assay. Cultures were performed in 96-well tissue culture plates in a final volume of 200 μl/well of RPMI 1640 supplemented with 10% FCS plus additives as described above. T cell hybridomas (5×10^4 or 10^5/well) were cultured for 24 h in the presence of various antigens and APCs. In experiments with inhibitors, APCs were pretreated with inhibitor for 1 h followed by the addition of antigen followed by an additional 4 h of culture. APCs were washed and fixed by exposure to 0.5% paraformaldehyde for 10 min at 24°C, followed by three washes in serum-containing medium. Thioglycollate-induced peritoneal exudate cells were obtained through lavage of the peritoneal cavity of mice injected intraperitoneally 3–5 d previously with 2 ml thioglycollate broth. Peritoneal cells (5×10^4/well) were cultured for 1.5 h at 37°C to allow macrophages to adhere, and nonadherent cells were removed by washing before T cell addition. In some experiments, 4.5 μm goat anti–rabbit Dynabeads™ (Dynal, A.S., Oslo, Norway) were added to stimulate phagocytosis (38). Lymphokine production, reflecting T cell activation, was quantified by measuring proliferation of the IL-2–dependent T cell line, CTL-L. Culture supernatants (100 μl) were transferred to flat-bottomed, 96-well tissue culture plates, freeze–thawed once, and cultured with 10^4 CTL-L cells/well for 24 h. Each well was pulsed with 1 μCi of [^{3}H]thymidine during the final 18 h of culture. All experiments were performed in triplicate, and data are representative of at least two independent experiments.

Results

In multiple independent fusions, we have isolated T cell hybridomas from pork insulin–primed nonresponder H-2^b mice that specifically recognize insulin but appear to be MHC-unrestricted based on the observation that they can be stimulated in the presence of MHC-mismatched APCs. These hybridomas express α/β TCRs, and CD8 is expressed only if a $CD8^+$ fusion partner is used to generate the cell lines. They do not express CD4 or γ/δ TCRs (data not shown). The T cells might recognize insulin peptides presented by nonpolymorphic MHC proteins, peptides bound to an alternative site in MHC class II molecules, or they may be stimulated through a mechanism completely independent of MHC molecules. The possibility that insulin may directly stimulate the T cells through hormonal receptors or other means was excluded because insulin does not activate the T cells in the absence of accessory cells (see below). We reported previously that insulin B chain peptides can bind to an alternative site overlapping the SEB-binding site outside of the peptide-binding groove in multiple different class II proteins (39). Therefore, the potential requirement for MHC class II molecules was addressed by evaluating the capacity of spleen cells from class II–deficient mice to present insulin to representative T cell hybridomas. As expected, these cells are unable to present insulin to control, IA^b-restricted T cells (Fig. 1 *A*). In contrast, the antigen presentation activity of class II–negative spleen cells was equal to that of wild-type B6 cells in experiments with MHC-unrestricted T cells (Fig. 1 *B*). The potential role of MHC class I molecules was investigated using spleen cells from β_2m knockout mice, which were observed to present insulin to the IA^b-restricted but not the unrestricted T cells (Fig. 1, *C* and *D*). These results suggested that the unrestricted T cells recognize insulin peptides presented by a nonpolymorphic β_2m-dependent class I or class I–like molecule.

The family of β_2m-associated class I–related molecules includes the classical class Ia proteins K, D, and L, class Ib proteins encoded in the Q, T, and M regions of the MHC, and molecules such as CD1 that are not encoded in the MHC. Experiments with APCs from a panel of inbred mice demonstrated a correlation between the identity of alleles encoded in the T region with the capacity to present insulin to Pb13 and 6C5 T cell hybridomas (Fig. 2). Antigen presentation did not correlate with class Ia loci or the Q or M regions. The restriction element was definitively mapped using congenic strains differing only in the T region. APCs from A/J-Tla^b and B6-Tla^b (T^b) mice presented insulin to 6C5 T cells, whereas those from congenic T^a strains did not (Fig. 2 *B*). B6-Tla^a spleen cells were fully capable of presenting insulin to control IA^b-restricted B1A4 T cells, ruling out any intrinsic defect in the general capacity to present insulin to T cells.

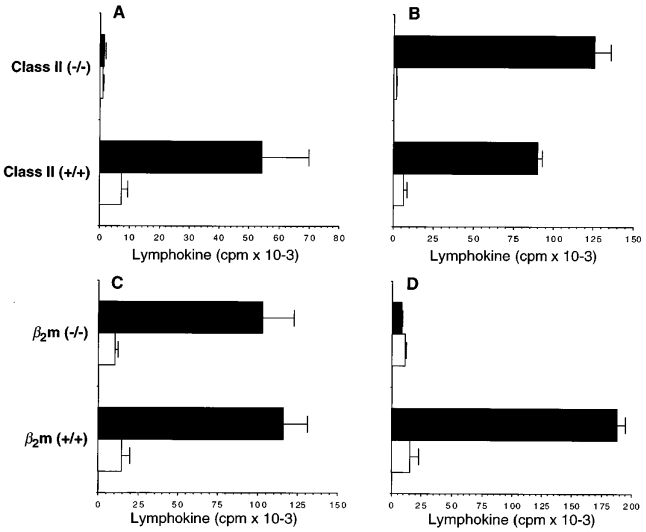

Figure 1. MHC class I– and class II–dependent recognition of insulin. T cell hybridomas (*A*) Pb1.4, (*B*) Pb13, (*C*) B1A4, and (*D*) 6C5 (10^5/well) were cultured with 10^6 spleen cells in the presence (*black bars*) or absence (*white bars*) of 30 μM pork insulin. Splenic APCs were obtained from β_2m-deficient, MHC class II–deficient, or wild-type B6 mice. Results represent lymphokine production measured as described in Materials and Methods. Pb1.4 and B1A4 are representative of MHC class II–restricted T cells, whereas Pb13 and 6C5 are representative of β_2m-dependent T cells isolated from pork insulin–primed B10 mice.

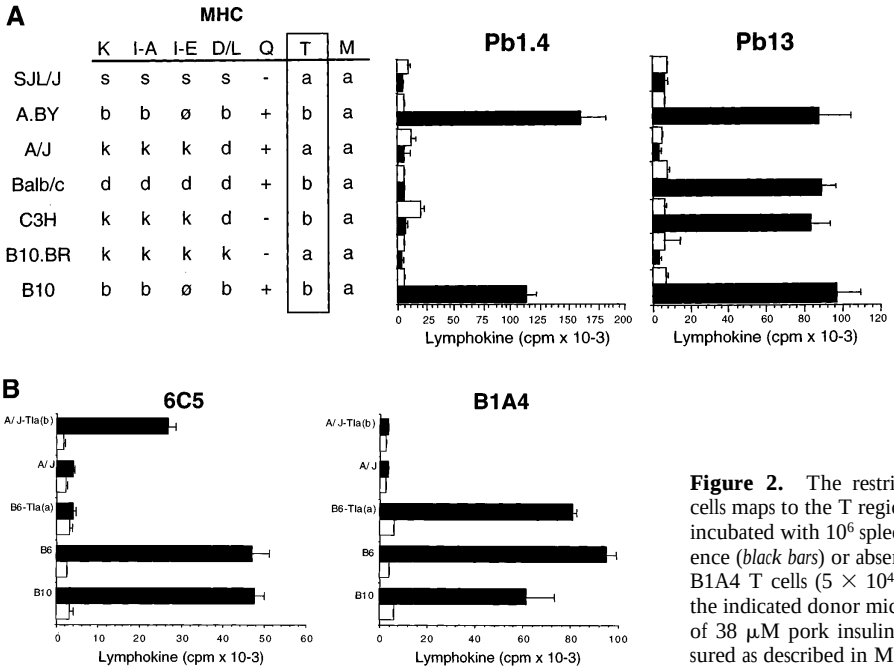

Figure 2. The restriction element recognized by β_2m-dependent T cells maps to the T region. (*A*) Pb1.4 or Pb13 T cells (5×10^4/well) were incubated with 10^6 spleen cells from the indicated donor mice in the presence (*black bars*) or absence (*white bars*) of 38 μM pork insulin. (*B*) 6C5 or B1A4 T cells (5×10^4/well) were incubated with 10^6 spleen cells from the indicated donor mice in the presence (*black bars*) or absence (*white bars*) of 38 μM pork insulin. Results represent lymphokine production measured as described in Materials and Methods.

The T region includes at least five genes with open reading frames that are transcribed or encode cell surface proteins (2). TCR γ/δ T cell lines can recognize the $T22^b$ gene product (40, 41). Products of the related T3/T18 genes can be recognized by TCR α/β cytotoxic T cells and act as transplantation antigens when expressed as transgenes under the control of the H-$2K^b$ promoter (42, 43). The T23 gene (formerly known as gene 37) encodes Qa-1, which can be recognized by alloreactive TCR α/β CTLs (10–13, 17, 18). Recent work also demonstrates that Qa-1^b can present antigen(s) from the intracellular pathogen *L. monocytogenes* to α/β CTLs (24). We used transfected cell lines to investigate the possibility that Qa-1^b was the restriction element recognized by our β_2m-dependent, insulin-specific T cells. The Qa-1^b–expressing L cell transfectant, L-g37, but not control transfectants, presented insulin to 6C5 T cells (Fig. 3 *A*). L-g37 did not present insulin to control, IA^b-restricted B1A4 T cells. The role of Qa-1^b was confirmed using transient Cos cell transfectants (Fig. 3 *B*), which ruled out the possibility that we had inadvertently selected cells expressing Qa-1–unrelated molecules required for stimulation of 6C5 T cells during isolation of the cloned, stably transfected L-g37 cell line. We conclude that the unrestricted T cell hybridomas isolated from pork insulin–primed H-2^b mice actually recognize insulin presented by the oligomorphic class Ib protein, Qa-1^b.

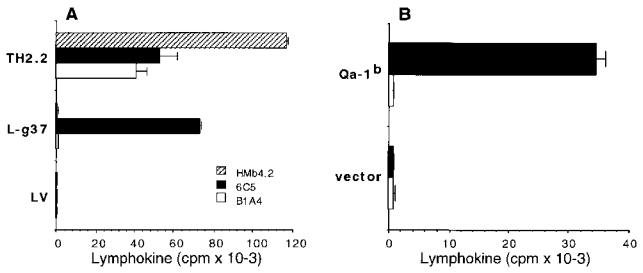

Figure 3. The restriction element recognized by β_2m-dependent T cells is Qa-1^b. (*A*) T cell hybridomas (10^5) were cultured with 10^5 TH2.2 B cells, Qa-1^b–expressing (*L-g37*) L cell transfectants, or vector control (*LV*) transfectants in the presence of 30 μM myoglobin (HMb4.2) or pork insulin (6C5 and B1A4). (*B*) Cos cells were transiently transfected with pCDNA3.0 alone (*vector*) or pCDNA3.0 containing cDNA encoding Qa-1^b. After 24 h, transfected cells (10^4) were cultured with 6C5 T cells (5×10^4) in the presence (*black bars*) or absence (*white bars*) of 30 μM pork insulin. Results represent lymphokine production measured as described in Materials and Methods.

The determinant specificity of pork insulin–induced IA^b- and Qa-1^b–restricted T cells was investigated (Fig. 4, and data not shown). Insulin contains a 30 amino acid B chain peptide that is linked through two disulfide bonds to a 21 amino acid A chain. The IA^b-restricted T cells were found to recognize a determinant within residues 17–30 of the B chain. The Qa-1^b–restricted T cells fully cross-react with beef insulin, which differs from pork insulin by two residues in the A chain loop that is formed by a disulfide bond between cysteines A6 and A11. The conclusion that the COOH terminus of the B chain is a critical component of the determinant was suggested by the observation that the T cells do not respond to desoctapeptide insulin, a tryptic peptide missing the COOH-terminal eight amino acids of the B chain. The antigenicity of sulfonated B chain and a synthetic B(1-30) peptide demonstrated that the determinant is fully localized in the B chain. The chemical status of the cysteine side chains does not appear to be important, because performic acid–oxidized insulin is antigenic. The B1 α amino group and the B29 ϵ amino group are not essential, since biotin-*N*-hydroxysuccinimide derivatives re-

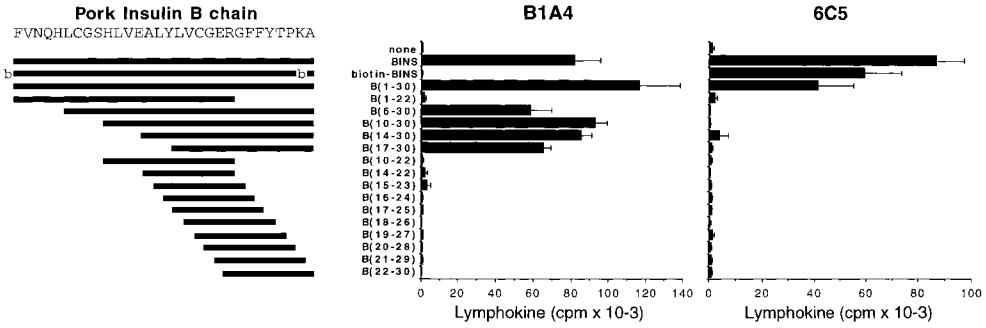

Figure 4. Insulin B chain specificity of IA^b- and Qa-1^b–restricted T cells. T cells (10^5/well) were incubated with 10^5 TH2.2 cells (B1A4) or 10^6 B10 spleen cells (6C5) and 30 μM of the indicated antigen. Results represent lymphokine production measured as described in Materials and Methods.

tain activity. By contrast, IA^b-restricted B1A4 cells do not respond to biotin-insulin, suggesting that the B29 Lys is a critical component of this determinant.

The only previously characterized peptide determinant recognized by Qa-1–restricted T cells is derived from the leader sequence of MHC class Ia D or L molecules (18). The Qdm nonamer (AMAPRTLLL) optimally stimulates a large fraction of Qa-1^b–restricted alloreactive CTLs. Peptide binding experiments with substituted Qdm analogues suggest that P2 and P9 may be dominant anchor positions (44). These findings support the assumption that the peptide specificity of Qa-1 is similar to class Ia proteins, with preference for nonamers bearing appropriate residues at key anchor positions. Given this assumption, we were surprised to find that none of a set of overlapping nonameric peptides from the COOH terminus of the pork insulin B chain stimulated Qa-1^b–restricted T cells (Fig. 4). We considered the possibility that the T cells preferentially recognize longer peptides. Aldrich et al. (18) reported that 10- and 11-mer Qdm derivatives extended at the COOH terminus stimulate alloreactived CTLs almost as well as the nonamer. However, none of a set of NH_2-terminally truncated B chain peptides beginning with B(6-30) stimulate our Qa-1^b–restricted T cells (Fig. 4). Thus, it appears that the determinant recognized by these cells includes much if not all of the full 30 amino acid B chain.

Given the near ubiquitous expression of the insulin hormonal receptor, it was important to rule out the possibility that the T cells recognize a determinant unrelated to insulin that is induced through the hormonal activity of insulin. This possibility was unlikely, because isolated insulin B chain has no hormonal activity (45) while retaining a capacity to stimulate the T cells. To further address this issue, we evaluated the antigen presentation activity of a hepatocyte cell line derived from IR-deficient mice generated by targeted gene disruption (35). The antigen presentation activity of IR-negative cells was equal to that of an IR-positive control hepatocyte cell line (Fig. 5). We conclude that the T cells directly recognize an insulin B chain determinant presented by Qa-1^b.

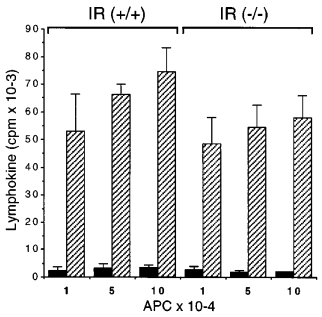

Figure 5. Antigen presentation by $IR^{-/-}$ APCs. 6C5 T cells (5×10^4/well) were cultured with hepatocyte cell lines, generated from $IR^{-/-}$ and $IR^{+/+}$ littermates, in the absence (*black bars*) or presence of 20 μM beef insulin (*striped bars*). Results represent lymphokine production measured as described in Materials and Methods.

The capacity of Qa-1^b class I molecules to present exogenous insulin suggests an unconventional antigen processing pathway. Several studies have demonstrated that subpopulations of macrophages (46–48) and dendritic cells (49–51) have the capacity to present peptides derived from internalized exogenous proteins via MHC class I molecules (52, 53). In some situations, internalized proteins somehow gain access to the cytoplasm and are processed by components of the conventional class I pathway (38, 48, 54, 55). In other situations, noncytosolic mechanisms are responsible for presentation of exogenous antigen by class I molecules (55–57). The role of the cytosolic MHC class I pathway in presenting insulin to Qa-1^b–restricted T cells was investigated using APCs from transporters associated with antigen processing (TAP)-deficient mice. TAP-deficient macrophages were observed to present insulin to Qa-1^b–restricted T cells almost as efficiently as control macrophages (Fig. 6 *A*). Similar results were obtained with unfractionated splenic APCs. The conclusion that Qa-1^b uses a noncytosolic pathway for presentation of insulin was further supported by the observation that chloroquine completely inhibits antigen presentation (Fig. 6 *B*). Brefeldin A (BFA) also inhibits presentation, probably by depleting the post-endoplasmic reticulum (ER) pool of Qa-1^b molecules. The cellular turnover rate of Qa-1^b has been reported to be greater than that of class Ia molecules (15). The cysteine protease inhibitor, leupeptin, did not inhibit presentation. Leupeptin inhibits the class II antigen presentation pathway primarily by inhibiting the proteolytic release of invariant chain from MHC class II molecules. It is noteworthy that the response of Qa-1^b–restricted, insulin-specific T cells to antigen-pulsed APCs is always substantially lower than that observed when antigen is present continuously in the T cell culture. It is possible that B chain–Qa-1^b complexes are short-lived and rapidly depleted after antigen removal.

Invariant chain has been shown to associate with a fraction of MHC class I molecules, targeting them to endo-

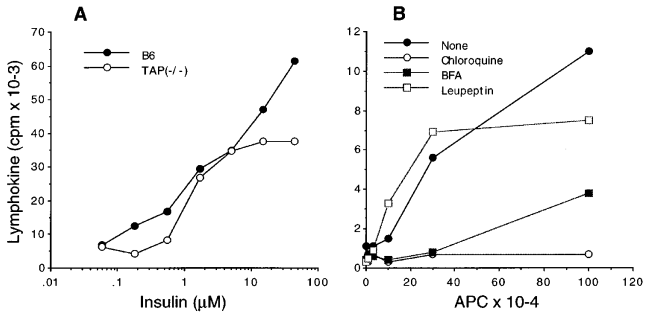

Figure 6. TAP-independent, chloroquine-sensitive presentation of insulin to Qa-1^b–restricted T cells. (*A*) 6C5 T cells (10^5/well) were cultured with various concentrations of antigen in the presence of peritoneal macrophages from TAP$^{-/-}$ or TAP$^{+/+}$ B6 mice and 10^8 1 μm latex beads to stimulate phagocytosis. (*B*) B10 spleen cells were preincubated with no inhibitor, 15 μg/ml chloroquine, 5 μg/ml BFA, or 200 μg/ml leupeptin for 1 h at 37°C. After addition of pork insulin (38 μM), the cultures were continued for 4 h and the cells were fixed with paraformaldehyde. Various numbers of treated spleen cells were cultured with 6C5 T cells (5×10^4), and lymphokine production was measured as described in Materials and Methods.

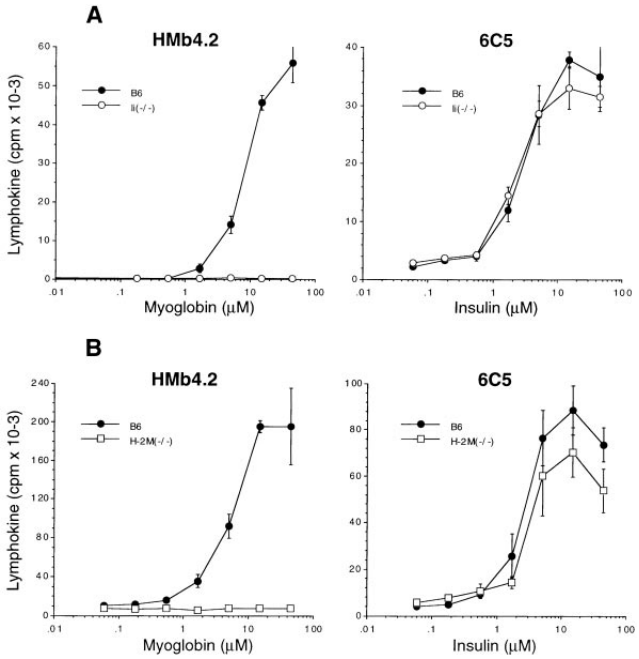

Figure 7. Invariant chain and H-2M are not required for presentation of insulin to Qa-1^b–restricted T cells. (*A*) HMb4.2 and 6C5 T cells (5×10^4/well) were incubated with various concentrations of antigen and 5×10^5 spleen cells from Ii$^{-/-}$ or Ii$^{+/+}$ B6 mice. (*B*) T cells (5×10^4/well) were incubated with various concentrations of antigen and 5×10^5 spleen cells from H-2M$^{-/-}$ or H-2M$^{+/+}$ B6 mice. Lymphokine production was measured as described in Materials and Methods.

somal compartments (58). Given evidence for a TAP-independent, chloroquine-sensitive endosomal processing pathway in presentation of insulin by Qa-1^b, we evaluated the antigen presentation activity of spleen cells from invariant chain–deficient mice (Fig. 7 *A*). Presentation of myoglobin to control IA^b-restricted HMb4.2 T cells was abrogated in the absence of invariant chain. In contrast, invariant chain expression had no effect on presentation to 6C5 T cells. H-2M catalyzes peptide-exchange reactions in MHC class II molecules localized in endosomal compartments (26, 59, 60). The possibility that H-2M can catalyze peptide exchange in class I molecules has not been addressed. We observed no significant difference in the antigen presenting activity of H-2M–deficient and control spleen cells in experiments with 6C5 T cells (Fig. 7 *B*). In contrast, control but not H-2M–negative APCs presented myoglobin to HMb4.2 T cells. In preliminary in vitro experiments, we found no evidence that the human homologue of H-2M, HLA-DM, can catalyze peptide-exchange reactions in purified MHC class Ia proteins (data not shown). We conclude that invariant chain and H-2M are not required for presentation of insulin to Qa-1^b–restricted T cells.

The cytosolic and noncytosolic exogenous class I antigen processing pathways are selectively localized in macrophages (46–48) and dendritic cells (49–51). MHC class II–negative cells, T cells, and B cells in general are unable to present exogenous protein determinants to class I–restricted T cells (47, 52). Exceptions have been reported, but they appear to be associated with specialized forms of antigen or specialized mechanisms of antigen uptake (61–63). We found that a variety of nonphagocytic cell lines present insulin to Qa-1^b–restricted T cells, with an efficiency similar to macrophages or spleen cells (Fig. 8). Antigen presentation activity correlated with Qa-1^b expression as measured by flow cytometry. 6C5 T cells do not express cell-surface

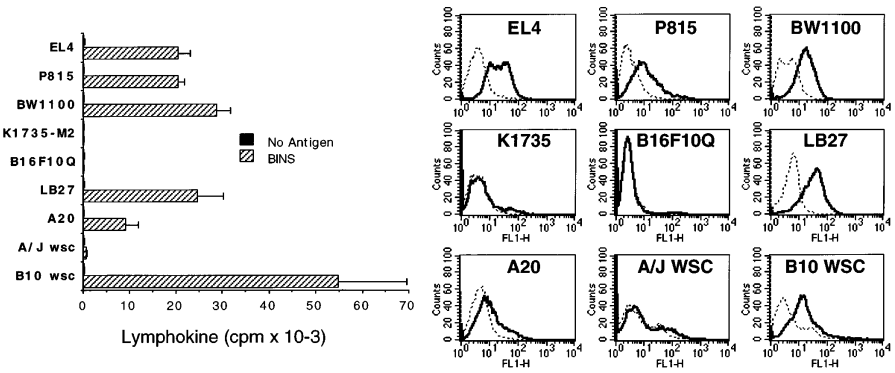

Figure 8. The antigen processing pathway for presentation of insulin by Qa-1^b is active in a variety of cell lines. 6C5 T cells (10^5/well) were incubated with the indicated cell lines (10^5/well) or spleen cells (10^6/well) in the presence or absence of 20 μM beef insulin (*BINS*). Lymphokine production was measured as described in Materials and Methods. The APCs were analyzed for cell surface Qa-1^b expression by flow cytometry using Qa-1–specific IgG (*solid lines*) or control rabbit IgG (*broken lines*).

Qa-1^b (data not shown), explaining their inability to respond to insulin in the absence of APCs.

The previously characterized exogenous class I antigen processing pathways are strongly stimulated by phagocytosis (38, 57, 64). H-2K^b–restricted B3Z T cells respond weakly to OVA presented by peritoneal macrophages (Fig. 9). This response is markedly enhanced in the presence of 4.5-μm particles as described previously (38). By contrast, macrophages efficiently present insulin to Qa-1^b–restricted T cells in the absence of phagocytic stimuli, and the response is only slightly enhanced in the presence of 4.5-μm particles (Fig. 9). Thus, it appears that Qa-1^b has access to a novel antigen processing pathway that is constitutively active in a wide range of cell types and is not stimulated by phagocytosis.

Discussion

We describe the isolation and initial characterization of TCR α/β T cell hybridomas from H-2^b mice that recognize pork insulin presented by the nonclassical class I protein, Qa-1^b. The restriction element was localized in experiments with T region congenic mice and identified using transfected cell lines expressing Qa-1^b. Blocking experiments with polyclonal rabbit antisera confirmed that the T cells directly recognize Qa-1^b (data not shown). Experiments with insulin-derived peptides demonstrated that the determinant recognized by these cells is localized to the B chain of insulin. The antigen processing pathway used to present insulin to Qa-1^b–restricted T cells is inhibited by chloroquine and does not require TAP. In contrast to previously characterized pathways for presentation of exogenous antigens to class I–restricted T cells, this pathway is constitutively active in a wide variety of cell types and it is not stimulated by phagocytosis.

The dominant peptide, Qdm, recognized by Qa-1^b–restricted TCR α/β alloreactive T cells is derived from the leader sequence of D or L MHC class Ia proteins (18). The natural form of this peptide isolated by acid extraction of Con A–activated lymphoblasts is a nonamer, and it is the major peptide associated with Qa-1^b in these cells (18, 19). Similar results were obtained from analysis of peptides eluted from purified chimeric class I molecules expressing the α1 and α2 domains of Qa-1^b and the α3 domain of D^b expressed in L cell transfectants (20). The limited heterogeneity of Qa-1^b–associated peptides may reflect a relative shortage of peptides in the ER with the appropriate sequence motif, or it could result from unknown constraints on the mechanism of peptide loading by Qa-1 molecules. However, the dominance of Qdm is consistent with the idea that Qa-1^b preferentially binds nonameric peptides similar to MHC class Ia proteins. Therefore, it is surprising that we were unable to identify short peptides from the insulin B chain that could stimulate our Qa-1^b–restricted T cells. It is clear that the COOH terminus of the B chain is essential for T cell activation. Desoctapeptide insulin and B(1-22), missing the eight COOH-terminal residues of the B chain, have no activity. However, the NH_2 terminus of the B chain also appears to have residues required for T cell activation. B(6-30) and shorter NH_2-terminally truncated B chain peptides do not stimulate the Qa-1^b–restricted T cells. It remains possible that the B chain is further processed in APCs, and that a covalently modified peptide represents the final species that associates with Qa-1^b.

The possibility that Qa-1^b may be especially suited to bind and present peptides of unusual length cannot be excluded. Peptide binding assays with Qa-1^b–expressing cells have been used to identify anchor residues in the Qdm sequence (20, 44); however, the capacity of longer peptides to bind Qa-1^b has not been directly examined. Aldrich et al. (18) reported that 8-, 10-, and 11-mer variants differing from Qdm at the COOH terminus stimulate alloreactive CTLs with only slightly reduced potency, whereas 7- and 12-mer peptides are 50–100-fold less potent but still demonstrate substantial activity in cytotoxicity assays. Longer peptides were not tested. Imani and Soloski (22) demonstrated that a tryptic digest of *M. bovis* 65-kD heat shock protein, containing peptides of various lengths, stabilizes cell surface Qa-1^b molecules. The determinant(s) recognized by recently described Qa-1^b–restricted, *L. monocytogenes*–specific CTLs has yet to be defined (24). Qa-1^b (and Qa-1^a) differ from other class I molecules by serines at positions 143 and 147 instead of threonine and tryptophan, respectively (15, 16, 65). These positions represent two of four highly conserved residues in the F pocket that form hydrogen bonds with the COOH terminus of class I–bound peptides, limiting their length and contributing substantially to the stability of the complexes (66). The consequence of these substitutions on the binding specificity and stability of Qa-1^b–peptide complexes is unknown, but it is possible that interactions involving the COOH terminus of bound peptide play a smaller role in Qa-1 than other class I molecules. Mouse CD1 preferentially binds peptides 14–24 amino acids in length to form complexes that can be recognized specifically by TCR α/β CTLs, providing an example of a class I–related molecule that binds long peptides

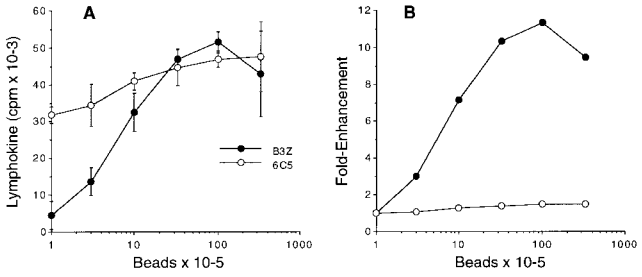

Figure 9. Phagocytic stimulus enhances the processing and presentation of OVA but not insulin by peritoneal macrophages. (*A*) B3Z (OVA-specific, K^b-restricted) or 6C5 T cells (5×10^4/well) were cultured with 5×10^4 thioglycollate-elicited peritoneal macrophages and 40 μM antigen in the absence or presence of 4.5 μm Dynabeads™ as indicated. Lymphokine production was measured as described in Materials and Methods. (*B*) The data were replotted to indicate the relative enhancement of T cell responses observed in the presence of beads.

(67). In addition, the three-dimensional structure of a peptide extending out of the COOH-terminal end of the peptide-binding site of a class Ia protein has been reported (68). The conserved residues that form hydrogen bonds with the peptide NH_2 terminus in class I molecules are conserved in Qa-1 (15, 16, 65). Thus, if the insulin B chain extends out of the NH_2-terminal end of the peptide-binding site in Qa-1^b, the complex is likely to be very unstable with a rapid rate of dissociation. This possibility must be considered because insulin derivatives with a chemically modified B chain α amino group retain activity in T cell assays. In addition, antigen-pulsed APCs stimulate substantially lower T cell responses compared with those observed in the continuous presence of antigen. This suggests that the relevant peptide complexes may be short-lived. Vidovic et al. (21) have described a TCR γ/δ T cell hybridoma that recognizes synthetic copolymer poly($Glu^{50}Tyr^{50}$) presented by Qa-1^b on the surface of fixed APCs. This polymer has been shown to stabilize cell surface Qa-1^b molecules (22). Soloski et al. (13) demonstrated that radioiodinated poly($Glu^{50}Tyr^{50}$) binds specifically to Qa-1^b on L cell transfectants without size selection, ruling out the possibility that short peptides contaminating the polymer preparation are responsible for the observed activity. Thus, several observations support the possibility that Qa-1^b may be able to bind and present unusually long peptide antigens.

The results of experiments with inhibitors and APCs from TAP knockout mice suggest that a noncytosolic processing pathway is used to present insulin to Qa-1^b–restricted T cells. Chloroquine completely inhibited antigen presentation, implicating endosomal compartments in the processing pathway. APCs from TAP-deficient animals were almost as efficient as cells from control mice in antigen presentation. The slightly reduced activity of TAP-deficient APCs can be explained by a reduction in the quantity of Qa-1^b–peptide complexes assembled in the ER and competent for export to the cell surface or endosomal compartments. Formation of Qa-1^b–Qdm complexes is TAP-dependent (69). Since this is likely to be the major peptide complex assembled in the ER (19, 70), one would expect a reduction in Qa-1 transport in TAP-negative APCs. Biochemical evidence has also been obtained demonstrating reduced maturation and transport of Qa-1^b molecules in activated T cells from TAP-negative mice (13). However, a substantial fraction of alloreactive CTLs specific for Qa-1^b recognize TAP-independent determinants (69), and Qa-1^b transport is only partially inhibited in TAP-deficient spleen cells (13). Thus, a cohort of Qa-1^b molecules should still be available for peptide-exchange reactions in post-ER compartments in TAP-deficient cells. This interpretation would also account for the observation that antigen presentation is substantially inhibited by BFA, which blocks transport to post-ER compartments. Qa-1^b has a very rapid turnover rate in cells (15, 22), and thus there may be a substantial reduction in the quantity of molecules available for exchange reactions in post-ER compartments after relatively short incubations with BFA.

Both cytosolic and noncytosolic pathways have been described for the presentation of exogenous protein antigens to class I–restricted T cells (52, 53). In general, these pathways are selectively active in macrophages (46–48) and a subpopulation of dendritic cells (49–51) but not B cells, T cells, or other class II–negative cell types. Therefore, it is striking that a wide variety of MHC class II–positive and –negative cell lines derived from different tissues are able to present insulin to Qa-1^b–restricted T cells with efficiencies similar to peritoneal macrophages or unfractionated spleen cells. Presentation of exogenous proteins by class I molecules in macrophages is markedly enhanced under conditions favoring uptake of antigen by phagocytosis (54, 56, 57, 64) or macropinocytosis (48). Reis e Sousa and Germain (38) demonstrated that antigen does not need to be physically coupled to particles to stimulate presentation of OVA to K^b-restricted T cells. This suggests that phagocytosis, by itself, activates the exogenous class I antigen processing pathway (38). Results presented here confirm that 4.5-μm particles strongly enhance the processing of soluble OVA by peritoneal macrophages for presentation to K^b-restricted T cells. The observation that particles have little effect on the presentation of insulin by macrophages to Qa-1^b–restricted T cells further distinguishes the exogenous antigen processing pathway used by Qa-1^b from that described previously for MHC class Ia molecules.

It is possible that Qa-1^b is selectively targeted to endosomal compartments, where it can participate in peptide-exchange reactions. The class I–related human CD1b protein is selectively targeted to endosomes through a tyrosine-based targeting motif in the cytoplasmic domain (6). A similar motif is present in the cytoplasmic domain of Qa-1^b, and experiments are in progress to evaluate the requirement for the cytoplasmic domain in stimulating insulin-specific T cells. Immunofluorescence analysis clearly demonstrates an endosomal localization pattern for CD1b (6). In contrast, Qa-1^b is predominantly localized in the ER and at the cell surface in a pattern similar to class Ia molecules (data not shown). Thus, if Qa-1^b is targeted to endosomes, only a fraction of the molecules reside in these compartments under steady-state conditions. Sugita and Brenner (58) demonstrated that a fraction of human class Ia molecules binds to the Ii in cells expressing this protein. The class I–invariant chain complexes were shown to be transported to endosomal compartments. We addressed the possibility that invariant chain may be required for targeting Qa-1^b to endosomes. However, splenic APCs from invariant chain–deficient mice were found to have no reduction in antigen presentation activity. It is possible that the intracellular trafficking pattern of Qa-1^b is similar to that of class Ia proteins, but that intrinsic features of the molecule make it particularly well suited to participate in peptide-exchange reactions. As noted above, Qa-1^b has a high turnover rate in cells (15, 22), which could result from rapid peptide dissociation and consequent denaturation of empty molecules. Kurepa and Forman (44) have reported that Qa-1^b–Qdm complexes are relatively stable, with a $t_{1/2}$ of $\sim$10 h at 4°C. However,

it is likely that these complexes are substantially less stable at 37°C. Given the assumption that Qa-1^b–Qdm complexes represent the most stable Qa-1^b–peptide complexes, it is likely that peptide-receptive Qa-1^b molecules are generated rapidly under physiological conditions.

The function of insulin-specific, Qa-1^b–restricted T cells remains to be determined. H-2^b mice are nonresponders to pork insulin, generating little if any antibody after immunization with this antigen (71). Beef insulin, differing from pork insulin by only two amino acids in the A chain, stimulates high-titered antibody responses in H-2^b mice. Weak secondary in vitro proliferation responses can be measured after immunization with pork insulin, demonstrating that pork insulin–reactive T cells can be induced in these animals. T cell hybridomas generated from pork insulin–primed B6 or B10 mice generated in our laboratory fall into two groups: $CD4^+$, TCR α/β–expressing cells that recognize B(17-30) presented by IA^b, and TCR α/β cells that recognize B chain presented by Qa-1^b. The Qa-1^b–restricted T cell hybridomas express CD8 only if a $CD8^+$ fusion partner is used in their generation. Loss of CD8 expression is commonly observed after fusion of $CD8^+$ T cells to BW5147 (72). Although it is likely that the Qa-1^b–restricted T cells express CD8 in vivo, we cannot rule out the possibility the hybridomas are derived from a $CD4^-CD8^-$ population. Anti-CD8 mAbs block antigen-induced stimulation of the $CD8^+$ hybridomas (data not shown). However, the observation that some hybridomas can recognize insulin presented by Qa-1^b in the absence of CD8 suggests that the repertoire contains high-avidity TCRs with this specificity. A preliminary analysis of five independent hybridomas suggests that TCR Vβ use is not restricted (data not shown). It is interesting to consider the possibility that the Qa-1^b–restricted T cells may somehow be involved in regulating the immune response to insulin in nonresponder mice. The poly($Glu^{50}Tyr^{50}$)-specific TCR γ/δ T cells reported by Vidovic et al. (21) were generated in nonresponder mice (73). In addition, Jiang et al. (23) have described TCR α/β CTLs responsible for the peripheral deletion of $CD4^+$ T cells that is observed after injection of SEB. These CTLs apparently recognize TCR Vβ determinants presented by Qa-1 (23). Thus, Qa-1 could have a special role in mediating regulatory interactions between T cells.

We thank our generous colleagues, Drs. Domenico Accili, Leszek Ignatowicz, Luc Van Kaer, Sasha Rudensky, and Nilabh Shastri for critical reagents. We are also grateful to Drs. Nicola Longo, Aron Lukacher, and Dominique Weber for their assistance and helpful discussions. Joe Moore provided excellent technical assistance.

This work was supported by National Institutes of Health grants AI-33614, AI-30554 (to P.E. Jensen), and AI-20922 (to M.J. Soloski).

Address correspondence to P.E. Jensen, Department of Pathology and Laboratory Medicine, Rm. 7309 WMB, Emory University School of Medicine, Atlanta, GA 30322. Phone: 404-727-3658; Fax: 404-727-5764; E-mail: pjensen@bimcore.emory.edu

Received for publication 19 May 1998.

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