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Cross-Antagonism of a T Cell Clone Expressing Two Distinct T Cell Receptors

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Summary

Inhibition of T cell activation can be mediated by analogs of the original antigenic peptide (TCR antagonists). Here, a T cell clone expressing two distinct TCR was used to investigate whether such inhibition involves an active mechanism by examining whether an antagonist for one TCR could influence responses stimulated by the other TCR engaging its agonist. Our results demonstrate functional cross-inhibition under these conditions involving the ability of antagonist: TCR interactions to diminish Lck enzymatic activity associated with the agonist-recognizing second TCR, apparently through enhancement of SHP-1 association with these receptors. Our findings reveal that inhibition of cellular responses by antagonists arises at least in part from active negative regulation of proximal TCR signaling and identify elements of the biochemical process.

Introduction

Engagement of the TCR with peptide/MHC complexes elicits biochemical intracellular signaling cascades that activate specific genes required for T cell proliferation, differentiation to effector function, and generation of memory cells (Crabtree and Clipstone, 1994; Weiss and Littman, 1994). A key early signaling event triggered by potent ligands is the activation of the src family kinase Lck, leading to tyrosine phosphorylation of the CD3 components ϵ and ζ (Straus and Weiss, 1992; Thome et al., 1995). With highly stimulatory ligands, the ratio of partially tyrosine phosphorylated ζ (p21 monomers in mouse, p32 dimers in human) to fully phosphorylated ζ (p23 monomers in mouse, p38 dimers in human) approaches 1:1 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Reis e Sousa et al., 1996; La Face et al., 1997; Hemmer et al., 1998; Kersh et al., 1998). Phosphorylation of the ITAM in these TCR-associated proteins is followed by SH2-domain–dependent binding of ZAP-70, which in the case of TCR containing fully phosphorylated (p23) ζ leads to the Lck-dependent phosphorylation and enzymatic activation of this Syk-family kinase (Chan et al., 1992).

This sequence of signaling events elicited by agonist peptide:MHC molecule ligands can be altered by subtle changes in the structure of either the antigenic peptide or the MHC molecule, which produce ligands characterized functionally as partial agonists or antagonists (Jameson and Bevan, 1995; Madrenas et al., 1995; Sette et al., 1996; Sloan-Lancaster and Allen, 1996). Antagonists are peptide:MHC molecule combinations that differ in one or more peptide or MHC residues from the agonist ligand for a TCR and that when offered together with suboptimal levels of agonist result in a reduction in T cell response (De Magistris et al., 1992; Racioppi et al., 1993; Jameson and Bevan, 1995; Dittel et al., 1997). Despite a failure to stimulate any measurable functional responses, pure antagonists do elicit a modified signaling response characterized by generation of p21ζ with little or no p23ζ, together with the recruitment of ZAP-70 without the phosphorylation or enzymatic activation of the bound ZAP-70 (Madrenas et al., 1995; Reis e Sousa et al., 1996; La Face et al., 1997; Hemmer et al., 1998).

Various mechanisms have been proposed to explain the function of antagonist ligands. Among these are inhibition of a TCR conformational change (Janeway, 1995), sterile binding to TCR resulting in a competitive reduction in occupancy by agonist (De Magistris et al., 1992; Daniels et al., 1999; Stotz et al., 1999) or in interference with serial engagement (Valitutti et al., 1995; Viola et al., 1997), consumption or sequestration of limiting signaling molecules in ineffective complexes (Torigoe et al., 1998), and a disruption of effective TCR or TCR:coreceptor oligomerization (Jameson and Bevan, 1995; Madrenas et al., 1997; Reich et al., 1997; Boniface et al., 1998). Recently, attention has been focused on observations of the low affinity of some antagonists for the TCR, especially an increased dissociation rate compared to effective agonists (Alam et al., 1996; Lyons et al., 1996; Rabinowitz et al., 1996; Kersh et al., 1998). This rapid dissociation has been proposed to result in partial signaling by early cessation of TCR engagement, disrupting the kinetics of signal development through the TCR (McKeithan, 1995). Although appealing, this hypothesis does not account for one report of increased affinity of an antagonist versus an agonist ligand measured using live cells (Sykulev et al., 1998).

Some of these hypotheses predict that for cells with two TCR of distinct peptide/MHC molecule specificity, inhibition of responses to a stimulatory ligand for one receptor would not be seen using an antagonist for the other TCR. The present study utilized a T cell clone expressing two distinct TCR to look for functional and biochemical cross-talk between the TCR upon exposure to agonist-antagonist combinations. Exposure of this dual TCR clone to the agonist of one TCR and an antagonist for the other led to reductions in cellular responses and kinase activity of the TCR interacting with agonist, despite the presence of increased Lck protein in these

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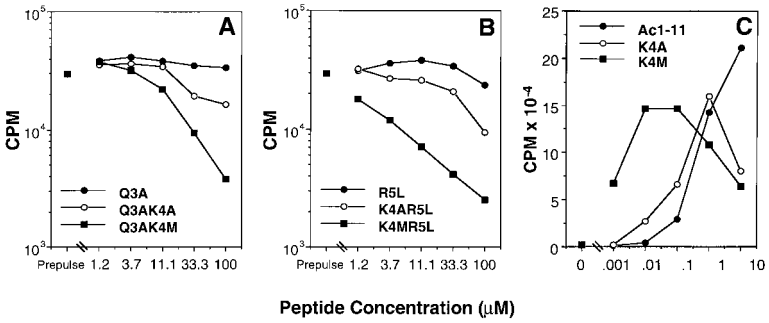

Figure 1. Dose-Dependent Peptide Antagonism of MBP TCR-Expressing Clones

(A and B) B10.PL splenocytes were prepulsed with K4M at 0.1 μg/ml for 2 hr at 37°C before coculture with clone 19 in the presence or absence of Q3A ([A], closed circle), Q3AK4A ([A], open circle), Q3AK4M ([A], closed square), R5L ([B], closed circle), K4AR5L ([B], open circle), or K4MR5L ([B], closed square) used at 1.2 to 100 μM. The prepulse level corresponds to proliferation of clone 19 in response to K4M prepulsed splenocytes in the absence of exogenously added peptide.

(C) The MBP 31 clone was cocultured with B10.PL (I-A^u) splenocytes in the presence of 1:10 dilutions of Ac1-11 (closed circle), K4A (open circle), or K4M (closed square) from 0.001 to 10 μM. Proliferation was measured by [^{3}H]TdR incorporation and presented as CPM.

receptors. This decrease in kinase activity was associated with enhanced levels of the tyrosine phosphatase SHP-1 in the TCR complexes. These data indicate that an active negative regulatory process contributes to the inhibition mediated by TCR antagonists and defines several of the key underlying events.

Results

Characterization of Antagonists for the D10 and MBP TCR

We have previously described the generation of CD4 T cell clones from the spleen of a tg mouse expressing the rearranged TCR α and β chain genes from the D10.G4.1 clone (D10) (Dittel et al., 1997) specific for hen egg conalbumin (CA) residues 134–146 (CA134-146) presented by the MHC class II molecule I-A^k (Kaye et al., 1983). In addition, we have previously characterized an antagonist peptide for the D10 TCR (E8A) in which the E at position eight of the peptide is substituted with A (Dittel et al., 1997) with the capacity to inhibit/antagonize both cell proliferation and cytokine secretion from Th1, Th2, and Th0 D10 TCR clones (Dittel et al., 1997).

To extend these studies to an examination of the mechanism of TCR antagonism, we searched for antagonists of a TCR that recognizes the acetylated N-terminal peptide of myelin basic protein (MBP) (Ac1-11) bound to I-A^u (Baron et al., 1993). For this purpose, we isolated T cell clones from the spleen of a tg mouse expressing the rearranged TCR α and β chains of an MBP-specific CD4 T cell clone (Hardardottir et al., 1995) and screened Ac1-11 peptides containing single amino acid substitutions in the TCR binding residues for generation of antagonist activity, as we have previously described for the D10 TCR (Dittel et al., 1997). Weak and variably active antagonist ligands were formed using peptides with A substituted at position 3 for the wild-type Q (Q3A) or L substituted at position 5 for R (R5L) (Figures 1A and 1B). In an effort to obtain more active inhibitors, we attempted to increase the affinity of the antagonist peptides for the I-A^u MHC class II molecule by substituting K at position four of Ac1-11 with A (K4A) or M (K4M) in the Q3A and R5L antagonist peptides. It has been reported that the K4A substitution increases the affinity of Ac1-11 for the I-Au MHC class II molecule more than 40-fold (Fugger et al., 1996), and the K4M peptide binds I-A^u even more avidly (Fairchild et al., 1993). Consistent with previously published results, the predicted increased affinities of K4A and K4M for I-A^u were evident in biological assays. Using a MBP TCR-expressing clone, T cell proliferation in response to the Ac1-11 peptide was first detected at 0.1 μM, whereas the sensitivity was increased to 0.01 and 0.001 μM with the K4A and K4M peptides, respectively (Figure 1C). Consistent with these proliferation data, the Q3AK4A and K4AR5L peptides were more potent in antagonizing responses dependent on this TCR than the Q3A and R5L peptides, though they were similar to each other in activity (Figures 1A and 1B), whereas the Q3AK4M (Figure 1A) and K4MR5L (Figure 1B) peptides generated the best antagonists for the MBP TCR. The expected specificity of action of the agonist and antagonist ligands of the MBP TCR was seen with cells bearing the D10 TCR by a lack of proliferation or antagonism in response to the Ac1-11, Q3A, Q3AK4A, Q3AK4M, R5L, K4AR5L, and K4MR5L peptides presented by I-A^k or I-A^u APC (data not shown). The reciprocal specificity was confirmed for the MBP TCR by lack of proliferation or antagonism in response to the CA134-146 or E8A peptides presented by I-A^k or I-A^u APC (data not shown).

Characterization of a Dual TCR Clone Expressing Both the D10 and MBP TCR

Having characterized two distinct TCR for their agonist and antagonist ligands, it was possible to design an approach to investigate whether antagonists' function may involve mechanisms other than interference with agonist-TCR engagement. CD4 T cell clones were generated from a mouse that expressed both transgenes and was heterozygous at the MHC (H-2^{kxu}). The clones stably expressed both TCR, as assessed by staining with the 3D3 (Kaye et al., 1983) and 19G (Baron et al., 1993) clonotypic mAbs, which are specific for the D10 TCR and MBP TCR, respectively (Figure 2A). Because TCR chains can cross-pair, although with distinct efficiencies (Saito et al., 1989; Couez et al., 1991), the specificity of the dual receptor D10/MBP 5-3 clone used in this study was analyzed using T cell proliferation and the CA134-146, Ac1-11, K4A, and K4M peptides. The D10/MBP 5-3 clone proliferates in response to the CA134-146 peptide in a dose-dependent manner when presented by I-A^k or I-A^{kxu} APC but not when this peptide is offered together with I-A^u APC (Figure 2B). Similarly, the D10/MBP 5-3 clone proliferates in a dose-dependent manner to the Ac1-11 peptide when presented by I-A^u or I-A^{kxu} APC but not when it is offered together with I-A^k APC (Figure 2B). To confirm that possible heterodimer

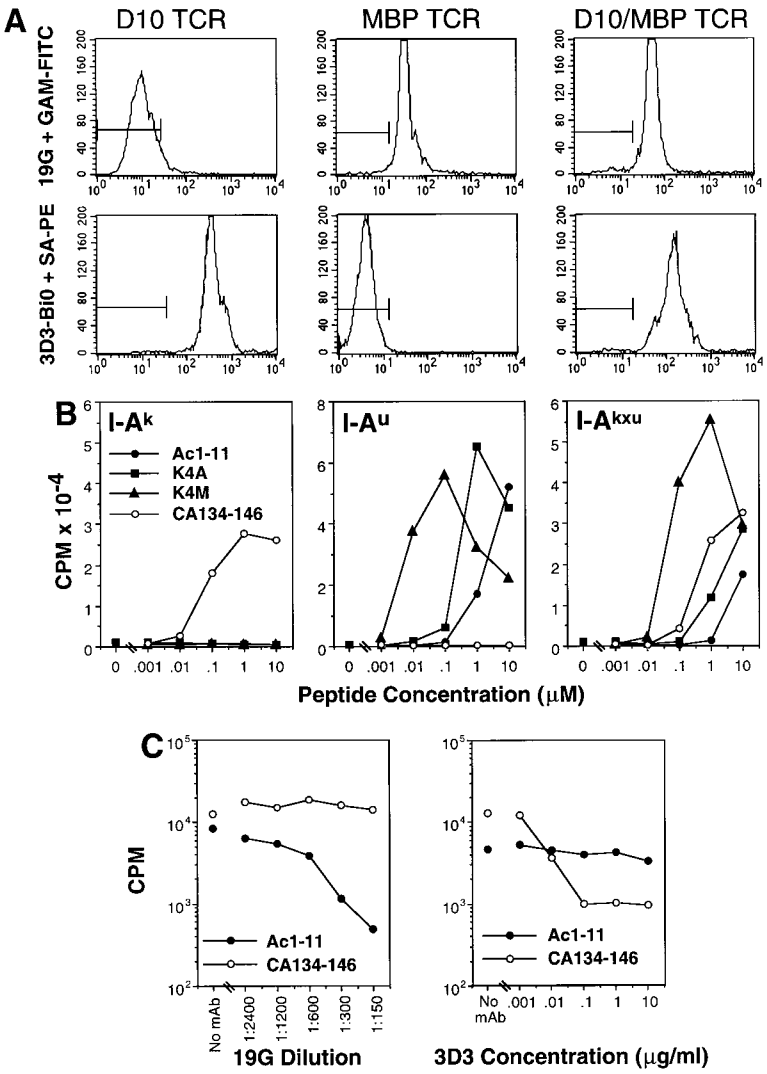

Figure 2. TCR Expression by and Response Characteristics of Single and Dual TCR Clones

(A) TCR expression of the D10 and MBP TCR on T cell clones obtained from D10, MBP, and D10/MBP TCR tg mice. The D10 Th2.3, clone 19, and D10/MBP 5-3 clones were stained for the expression of the D10 and MBP TCR using the TCR clonotypic mAbs 3D3 and 19G, respectively. Histograms represent fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. The horizontal bar in each histogram defines background staining.

(B) The D10/MBP 5-3 clone was cocultured with B10.BR ($I\text{-}A^k$) (left panel), B10.PL ($I\text{-}A^u$) (center panel), or with (B10.BR/B10.PL)F1 ($I\text{-}A^{kxu}$) (right panel) splenocytes in the presence of Ac1-11 (closed circle), K4A (square), K4M (triangle), or CA134-146 (open circle) offered at 0.001 to 10 μM.

(C) The D10/MBP 5-3 clone was cocultured with B10.PL splenocytes and 0.5 μg/ml Ac1-11 (open circles) or B10.BR splenocytes and 0.05 μg/ml CA134-146 (closed circles) in the presence of serial dilutions of 19G ascites (left panel) or purified 3D3 (right panel). Anti-Fc receptor mAb (10 μg/ml) was also added to each well. Proliferation was measured as for Figure 1.

formation between the TCR chains of the D10 and MBP TCR have no functional activity, we performed mAb inhibition studies using 3D3 and 19G. As shown in Figure 2C, proliferation of the D10/MBP 5-3 clone stimulated with $I\text{-}A^u$ APC presenting Ac1-11 was specifically inhibited with the 19G mAb but not 3D3. The reciprocal experiment gave a similar result with the 3D3 mAb but not 19G specifically inhibiting proliferation induced by CA134-146 presented by $I\text{-}A^k$ APC. Thus, as expected from the original T hybridoma fusion studies (Kappler et al., 1981), mixed peptide/MHC specificities are not seen using this dual TCR clone.

To further investigate the fine specificity of this dual TCR D10/MBP clone, we measured proliferation to the Ac1-11 analog peptides K4A and K4M. As with the MBP TCR clone shown in Figure 1B, proliferation to K4A and K4M was first detected at lower peptide concentrations than with Ac1-11 (Figure 2B). The sensitivity of the D10/MBP-dual TCR clone to Ac1-11, K4A, and K4M was reduced by approximately 10-fold when $I\text{-}A^{kxu}$ APC were used (Figure 2B). These data show that the D10/MBP 5-3 dual TCR clone responds to Ac1-11, K4A, and K4M in a similar manner as the MBP single TCR clone shown in Figure 1C. Likewise, the D10/MBP 5-3 clone also responds to CA134-146 similarly to D10 single TCR clones (Dittel et al., 1997).

Figure 3A shows that the proliferative response of the D10/MBP 5-3 clone is inhibited by the E8A antagonist upon stimulation with CA134-146, as reported for D10 clones (Dittel et al., 1997). Likewise, the response of the D10/MBP 5-3 clone is inhibited by the Q3AK4M antagonist upon stimulation with the K4A agonist (Figure 3D), similar to the single TCR MBP clone shown in Figure 1. Thus, both the D10 and MBP TCR in the D10/MBP 5-3 clone are fully functional and show the same behavior upon exposure to the previously characterized agonist and antagonist ligands as single TCR clones. The W7M and the HEL30-53 peptides are used as controls for binding to $I\text{-}A^k$ and $I\text{-}A^u$, respectively, and have no intrinsic ability to stimulate either the D10 or MBP TCR.

Antagonism Can Be Observed Using TCR-Engaging Ligands Involving Distinct MHC Molecules

This dual D10/MBP 5-3 T cell clone was then used to examine whether agonist stimulation through the D10 TCR could be inhibited by antagonist engagement of the MBP TCR. Functional cross-antagonism between two different TCR is demonstrated in Figure 3B by the

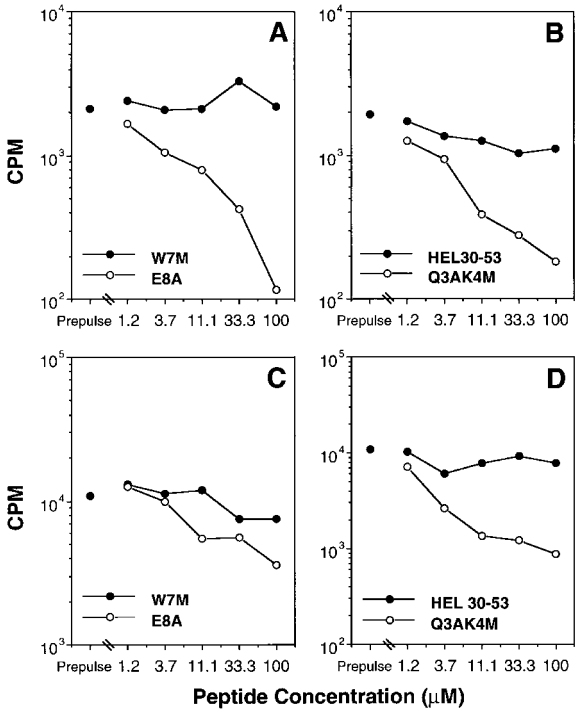

Figure 3. Dose-Dependent Peptide Antagonism of the D10/MBP 5-3 Clone

(B10.BR/B10.PL)F1 splenocytes were prepulsed with CA134-146 (10 μg/ml) (A and B) or K4A (40 μg/ml) (C and D) for 2 hr at 37°C before coculture with the D10/MBP 5-3 clone in the presence or absence of W7M ([A] and [C], closed circle), E8A ([A] and [C], open circle), Q3AK4M ([B] and [D], open circle), or HEL30-53 ([B] and [D], closed circle) offered at 1.2 to 100 μM. The prepulse level is proliferation in response to CA134-146 or K4A prepulsed APC in the absence of added peptide. Proliferation was measured as for Figure 1.

inhibition of the response of the D10/MBP 5-3 clone through the MBP TCR upon exposure to the Q3AK4M antagonist at the same time as stimulation through the D10 TCR with the CA134-146 agonist. The reciprocal experiment involving engagement of the D10 TCR with the E8A antagonist together with K4A agonist recognition through the MBP TCR showed similar cross-inhibition (Figure 3C).

To test whether the agonist and antagonist ligands must be presented together on the same APC or could be presented on separate APC, we performed antagonist assays using I-A^k, I-A^u, I-A^{kxu}, or a 1:1 mixture of I-A^k + I-A^u APC. Inhibition of a D10-TCR clone with E8A was achieved when CA134-146 prepulsed APC expressed I-A^k (Figure 4A) or I-A^{kxu} (Figure 4B) or when prepulsed I-A^k APC were mixed with I-A^u APC (Figure 4C). Using the D10/MBP 5-3 clone, E8A, but not Q3AK4M, inhibited proliferation to CA134-146 prepulsed I-A^k APC (Figure 4D). When I-A^{kxu} APC were prepulsed with CA134-146, as in Figure 3, inhibition was observed with both the E8A and Q3AK4M peptides (Figure 4E). In the case of I-A^{kxu} APC, both the agonist CA134-146 and the antagonist Q3AK4M have the capacity to be presented on the same APC (Figures 4B and 4E). When I-A^k APC prepulsed with the agonist CA134-146 peptide were mixed with I-A^u APC, E8A but not Q3AK4M mediated antagonism (Figure 4E). These data demonstrate that both ligands must be presented on the same APC in order to observe inhibition of responses mediated by an agonist-engaged TCR upon antagonist recognition by the other. Taken as a whole, these data show that competition for TCR binding cannot be the sole mechanism involved in antagonist function and further reveal that antagonists can inhibit responses by a TCR responding to agonist ligands of entirely distinct specificities.

TCR Proximal Tyrosine Phosphorylation Induced in a Dual TCR Clone after Stimulation of D10 or MBP TCR

The pattern of phosphorylation of TCR ζ and ϵ chains and the extent of phosphorylation and activation of ZAP-70 kinase have been established as reliable indicators of the quality of TCR interaction with peptide ligand presented by MHC molecules (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Reis e Sousa et al., 1996; La Face et al., 1997; Hemmer et al., 1998). To determine if the present characterization of peptide ligands by functional studies was consistent with these biochemical phenotypes, we examined the signaling responses of the D10 and the MBP TCR receptors after interaction with individual peptides presented by either I-A^k- or I-A^u-expressing fibroblasts (Figure 5). In agreement with the interpretation of the functional studies, the 5-3 dual TCR clone recognized K4M, K4A, and CA134-146 presented by the relevant MHC class II molecule as agonists and induced generation of both p21ζ and p23 (Figure 5). The ratio of p23/p21ζ and the intensity of phospho-ϵ and phospho-ZAP-70 elicited in response to CA134-146 were repeatedly lower when compared to the agonists formed by K4M and K4A, suggesting that the former peptide creates a low activity agonist ligand for the D10 TCR (Figure 5). Compared to other TCR:ligands pairs, even the K4M agonist shows a less than optimal p23/p21 ratio and production of a low level of phosphorylated ZAP-70 (Figure 5; data not shown). The signaling patterns seen with these stimulatory ligands were nevertheless clearly different from those induced by the peptides forming inhibitory ligands (antagonists). The latter triggered only partial phosphorylation of the ζ chain, generating almost exclusively p21-phospho-ζ (Figure 5). The intensity of p21ζ correlated with each antagonist ligand's potency in inhibiting agonist signaling (Figure 1), with the most effective functional antagonists Q3AK4M, K4MR5L, and E8A eliciting the strongest partial phosphorylation of ζ. Q3AK4A and K4AR5L, relatively less potent antagonists, induced only moderate p21ζ signals (Figure 5). Q3A and R5L induced ζ phosphorlation that was barely detectable over the signal present in control cells (Figure 5A), in accord with their very limited capacity to antagonize responses to agonist (Figure 1).

Early Transinhibition of Protein Tyrosine Kinase Activity in TCR Stimulated with Agonist in the Presence of Signals Generated by Antagonist

Having demonstrated that the agonist and antagonist ligands characterized here for the D10 and MBP TCR evoke intracellular signaling patterns predicted by previous studies, we asked whether these patterns were altered in quality and/or amount under conditions in which

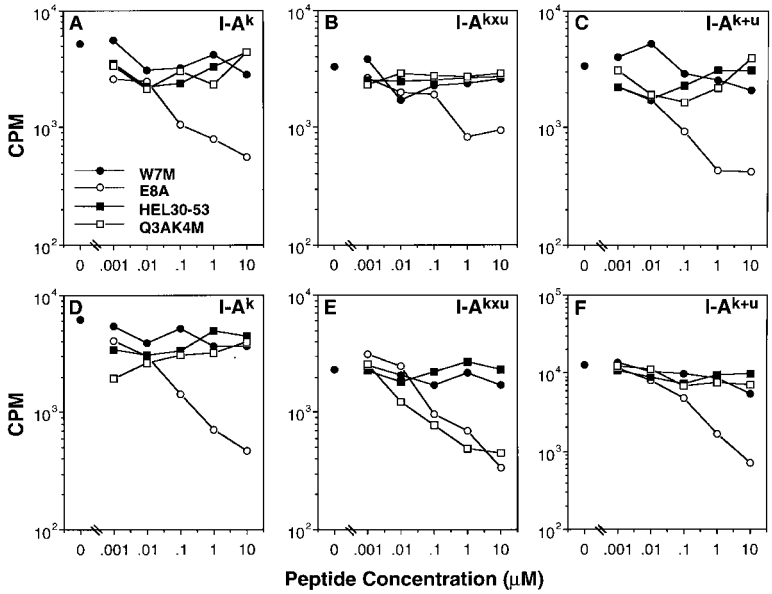

Figure 4. Dose-Dependent Peptide Antagonism of the D10.2.2 and the D10/MBP 5-3 Clone

B10.BR (I-A^k) and (B10.BR/B10.PL)F1 (I-A^{kxu}) splenocytes were prepulsed with CA134-146 for 2 hr at 37°C before coculture with the D10.2.2 and D10/MBP 5-3 clones in the presence or absence of W7M (closed circle), E8A (open circle), HEL30-53 (closed square), or Q3AK4M (open square) used at 1.2 to 100 μM. B10.BR splenocytes were prepulsed with 2.5 μg/ml (A, C, and D) or 5 μg/ml (F) CA134-146, and the (B10.BR/B10.PL)F1 splenocytes were prepulsed with 10 μg/ml (B and E) CA134-146. B10.BR (1×10^5) (A, C, D, and F) or 2×10^5 (B10.BR/B10.PL)F1 (B and E) splenocytes were cocultured with 2×10^4 clone cells. In (C) and (F), 1×10^5 B10.PL (I-A^u) splenocytes were used. The prepulse level is proliferation in response to CA134-146 prepulsed APC in the absence of added peptide. Proliferation was measured as for Figure 1.

functional transinhibition is seen, that is, when an antagonist ligand engaging one TCR inhibits the response to agonist seen by the second TCR of the 5-3 clone. D10 TCR complexes isolated from cells stimulated with agonist for this receptor in the absence or in the presence of antagonist for the MBP TCR were analyzed for associated protein kinase activity and for the presence of Lck. This is a key tyrosine kinase involved in phosphorylation of receptor-associated proteins and downstream signal propagation in T cells (Straus and Weiss, 1992). In cells stimulated with CA134-146 only, increased kinase activity was observed in the TCR complex along with a rise in the total level of TCR-associated Lck (Figure 6). The Lck associated with the D10 receptor in the resting state was primarily in the p56 mobility state, whereas after agonist exposure and during the peak of increase in kinase activity, both the p56 and p59 mobility isoforms were observed (Figure 6). p59 Lck is a serine phosphorylated form of Lck, presumably generated by the action of activated MAP kinase (Winkler et al., 1993). This change in Lck mobility thus indicates effective downstream signaling triggered by interaction of the D10 TCR with agonist ligand.

In contrast, kinase activity in the D10 TCR decreased

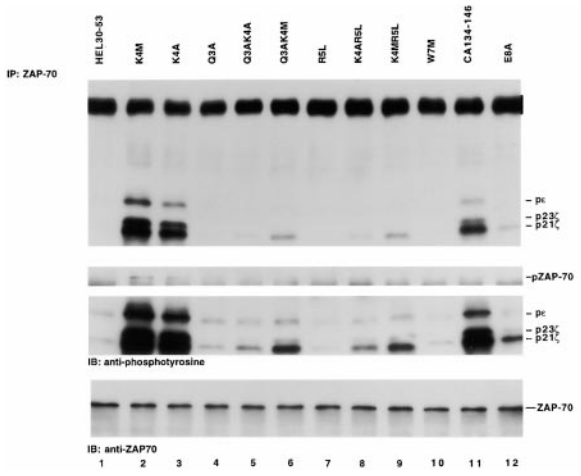

Figure 5. Early Signaling Induced in the Dual TCR T Cell Clone by Agonist and Antagonist Peptides

T cells were stimulated with the indicated peptides using pulsed I-A^u (lanes 1–9) or I-A^k (lanes 10–12) L cells for 5 min. Tyrosine phosphorylation of ZAP-70 and the ζ chain in anti-ZAP-70 immunoprecipitated complexes was visualized by anti-phosphotyrosine immunoblotting. The equal level of ZAP-70 in all samples is shown in the first panel. Different exposures of the ZAP-70 and the phosphorylated ζ chain are shown to allow comparison of the patterns seen under strong and weak stimulatory conditions.

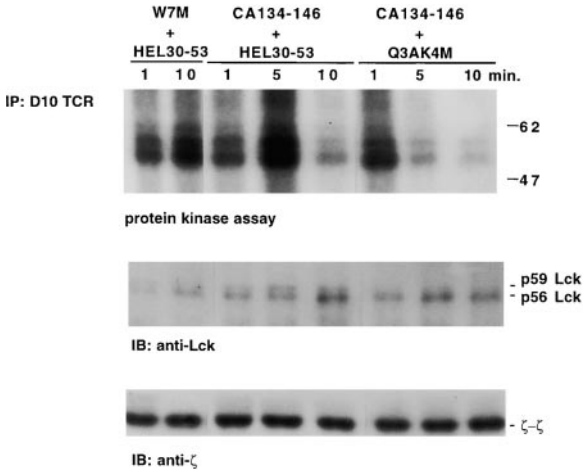

Figure 6. Simultaneous Presentation of Agonist and Antagonist Inhibits Tyrosine Kinase Activity in Agonist-Stimulated TCR in the Absence of Recruited Lck

T cells were stimulated for indicated periods of time using peptide-pulsed splenocytes from (B10.BR/B10.PL)F1 mice. Agonist CA134-146 was used at 10 μg/ml and antagonist Q3AK4M at 100 μg/ml. D10 TCR were isolated from stimulated cells and then analyzed for kinase activity (upper row), for the presence of Lck (middle row), and for ζ (lower row). All the data in Figure 6 are from the same experiment, blotted on the same membrane.

within the first 5 min in T cells stimulated with CA134-146 in the presence of the Q3AK4M antagonist for the MBP TCR, despite the fact that additional Lck was still recruited into the D10 TCR under these conditions (Figure 6). In contrast to what was observed using agonist alone, this recruited Lck consisted almost exclusively of the 56 kDa form, indicating limited downstream signaling through the MAPK pathway. Thus, the functional inhibition mediated in *trans* using antagonists with the dual TCR 5-3 clone was reflected in a very early change in the signaling properties of the agonist-engaged TCR, a change that involved interference with the enzymatic activity of recruited receptor-associated tyrosine kinase.

Infectious Antagonism: Antagonist-Engaged Receptors Induce SHP-1 Association with Bystander Unengaged Receptors

TCR recognition of peptide antagonists interfered with the signaling competency of distinct receptors engaged by agonist (Figure 6). This effect could not be explained by competition between TCR for the key initial protein tyrosine kinase Lck, because the total level of Lck integrated into agonist-engaging TCR is comparable in cells exposed to agonist either in the presence or in the absence of antagonist for the second TCR. These data were also inconsistent with simple competition models for antagonist function, given the distinct specificities of the two TCR involved and the gain in Lck. They seemed best explained by a model involving antagonist-mediated induction or recruitment into the TCR complexes of a negative regulator of tyrosine kinases, which in the case of the dual TCR clone as studied here would necessarily involve the spread of such a negative signal from the antagonist-engaged TCR to those recognizing agonist. We have recently found that antagonist engagement induces the rapid recruitment of one such inhibitory molecule to the TCR, namely the hematopoietic tyrosine phosphatase SHP-1, accompanied by a loss of Lck kinase activity in the affected TCR (I. S. et al., submitted). This previous study of SHP-1 recruitment involved cells with a single TCR specificity, however, and therefore could not distinguish between SHP-1 inhibition of only those TCR directly engaged by antagonist or a potential transinhibitory effect of SHP-1 on bystander TCR. The data on transinhibition of Lck activity in the dual TCR clone (Figure 7) suggested the later scenario as a more likely alternative. To look for "SHP-1 spreading" in the dual TCR clone, we stimulated the T cells with L cells expressing I-A^u presenting either agonist (K4M) or antagonist (Q3AK4M) peptides for the MBP TCR and analyzed total ζ phosphorylation (Figure 7A) and also the level of SHP-1 in the nonengaged D10 TCR (Figure 7B). A sustained rise in SHP-1 associated with the D10 TCR began within the first minute of exposure of the T cells to APC presenting antagonist for the MBP TCR. The ζ chain in the D10 TCR complex was not phosphorylated, consistent with absence of the I-A^k-presenting element in the system. This lack of tyrosine phosphorylated proteins in the anti-D10 immunoprecipitate also excludes the possibility that SHP-1 is part of MBP TCR and simply is coprecipitated with the D10 TCR, as such coprecipitation would result in the detectable presence of phospho-ζ (Figure 7A). SHP-1 was also

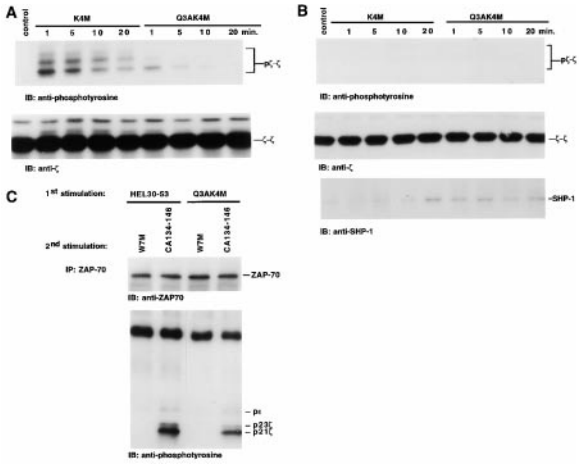

Figure 7. SHP-1 Is Recruited into Nonengaged Bystander TCR

T cells were stimulated with the indicated peptides using pulsed I-A^u L cells. Tyrosine phosphorylation of the ζ chain in immunoprecipitated total (A) and specific D10 TCR complexes (B) was visualized by anti-phosphotyrosine immunoblotting. SHP-1 in D10 TCR was detected by SHP-1 immunoblotting (B). Transient exposure to antagonist for the MBP TCR interferes with the subsequent response of the unengaged D10 TCR to agonist. T cells preexposed to I-A^u L cells in the presence or absence of antagonist Q3AK4M peptide were stimulated for 5 min with I-A^k L cells pulsed with 100 μg/ml CA134-146 peptide. Tyrosine phosphorylation and ZAP-70 protein in anti-ZAP-70 immunoprecipitated complexes were visualized by anti-phosphotyrosine and anti-ZAP-70 immunoblotting, respectively.

recruited into the D10 TCR after T cell stimulation with an agonist for the MBP TCR but only after 20 min of stimulation. This result is again consistent with our previous observation of the delayed kinetics of SHP-1 recruitment into TCR of agonist-stimulated cells (I. S. et al., submitted).

Infectious Spread of SHP-1 upon Antagonist Recognition Leads to Desensitization of Previously Unengaged TCR

A prediction of the TCR competition model for antagonist function (Daniels et al., 1999; Stotz et al., 1999) is that separating exposure of the T cell to antagonist and agonist in time should prevent inhibition. In a human T cell model, we have recently found that sequential exposure to antagonist and then agonist alone still led to response inhibition that correlated with the presence of SHP-1 in the antagonist-prestimulated TCR (I. S. et al., submitted). Based on these data, we expected that the infectious spread of SHP-1 to unengaged D10 TCR in cells preexposed to antagonist for the MBP TCR should reduce the subsequent signaling performance of the D10 TCR. To examine this possibility, the APC presenting Q3AK4M antagonist were separated from 5-3 cells after a first incubation that allowed extensive T cell contact with only antagonist-bearing APC over a prolonged time, to induce a sufficient degree of SHP-1 spreading. The recovered T cells were then exposed to APC presenting agonist for the D10 TCR. The phosphorylation elicited in antagonist-experienced cells was diminished compared to that of control cells (Figure 7C). This result

supports the view that inhibition by antagonists can be mediated by an active intracellular process distinct from receptor competition. The binding of SHP-1 to bystander TCR provides at least one explanation for, and is a mechanism consistent with, the molecular phenotype of the agonist-engaged TCR in cells also exposed to antagonist, namely accumulation of Lck in the TCR complex in the absence of kinase function.

Discussion

In this paper, we have described and studied the TCR signaling properties of a novel TCR clone derived from an F1 mouse expressing two well-characterized TCR. These receptors are specific for distinct agonist peptides bound to different alleles of the MHC class II A locus molecule. Variants of the antigenic peptides for each TCR were produced and characterized for their functional activity as TCR antagonists, as well as for their generation of the previously characterized altered pattern of proximal TCR-associated signaling (generation of p21 phospho-ζ and recruitment but not phosphorylation of ZAP-70). The antagonist ligands thus identified were then used with the dual TCR clone to test whether antagonist engagement by one TCR would inhibit responses stimulated by agonist recognition involving the other TCR. This is a critical test of several models proposed for the mechanistic basis of antagonist function, especially competition for receptor occupancy (De Magistris et al., 1992, Stotz et al., 1999, Daniels et al., 1999).

Cross-inhibition of responses induced by an agonist for one TCR in the presence of antagonist for the other was observed in both directions when the two types of ligands were present on the same APC. Such transinhibition was associated with accumulation of a kinase-inactive p56 form of Lck in the TCR recognizing agonist. In dual TCR cells exposed to antagonist alone, even TCR not recognizing added ligand and lacking detectable phosphorylation could be shown to be associated with the phosphatase SHP-1. Finally, transient exposure to antagonist for one TCR, followed sequentially by exposure to only agonist for the other receptor, revealed reduced phosphorylation responses consistent with the expected effect of preassociated SHP-1 on signaling by these agonist-engaged TCR. These observations suggest that antagonism is mediated, at least in part, by an active process distinct from either competitive inhibition of individual or serial agonist recognition events (De Magistris et al., 1992; Viola et al., 1997; Stotz et al., 1999) or from sequestration of the initiating tyrosine kinase (Torigoe et al., 1998). This negative regulatory process accompanying antagonist recognition can lead to inactivation of Lck in receptors engaging agonist, contributing to incomplete phosphorylation of TCR-associated proteins, interference with effective signal transduction, and reduced cellular responses.

In the presence of antagonist, there was a reduction in the appearance of the p59 form of Lck in association with the TCR complexes involved in agonist recognition. This isoform of Lck is generated by serine phosphorylation at residue 59 mediated by MAPK (Winkler et al., 1993) and is seen as a predominant form of the enzyme in TCR complexes triggered by agonist in the absence of antagonist (I. S. and R. N. G., unpublished data; Figure 6). In cells exposed to both CA agonist and MBP antagonist, the reduction in p59 Lck in TCR binding to agonist was correlated with a decrease in total Lck kinase activity associated with the D10 TCR (Figure 6). Antagonist exposure did not, however, inhibit the recruitment of Lck into the D10 TCR complex, as shown by the increase in the p56 form and by the equal levels of total Lck after 10 min in the presence or absence of antagonist (Figure 6). Thus, an antagonist does not change the kinetics of Lck recruitment, an early event in TCR signaling, but changes the nature of the signaling that follows by preventing or terminating Lck function.

The observed loss of Lck kinase activity can be accounted for by the recruitment of SHP-1 tyrosine phosphatase to the TCR complex. SHP-1 is tyrosine phosphorylated by Lck at a C-terminal site that is a consensus sequence for binding to the SH2 domain of Lck (Lorenz et al., 1994). Thus, Lck activation by antagonists (which explains the formation of p21ζ) would lead to a rise in the intracellular levels of such phosphorylated SHP-1, enhancing binding to Lck associated with both the antagonist-engaged receptors and other TCR, as we observe. SHP-1 has been associated with the regulation of TCR signaling in several prior studies. Overexpression of a dominant-negative form of SHP-1 enhances the sensitivity of the response of a T cell hybridoma or T cells in transgenic mice expressing this mutant phosphatase (Plas et al., 1996, 1999), and a molecular defect in SHP-1 in cells of motheaten mice results in prolonged thymocyte proliferative responses associated with constitutive tyrosine phosphorylation of TCR components, suggesting the absence of negative regulator of TCR signaling (Pani et al., 1996). Following stimulation with an agonist, SHP-1 is not visibly recruited to the TCR complex for 5–10 min, after which time the signaling pattern of the agonist-engaged TCR changes to one more characteristic of partial agonist/antagonist recognition (Figure 7; I. S. et al., submitted). Antagonist ligands induce much more rapid SHP-1 recruitment into the TCR complex, with readily detectable levels seen within the first minute of stimulation (Figure 7; data not shown). The promotion of SHP-1 binding to TCR induced by antagonist recognition is "infectious," spreading to surrounding agonist-engaged TCR or even TCR not exposed to any cognate peptide or MHC molecule, and hence presumably unengaged by ligand. The interference with signaling resulting from SHP-1 recruitment arises from limiting kinase activity and not from loss of kinase association with the TCR, thus differing from the sequestration of Lyn kinase concluded to underlie low-affinity ligand antagonism of high-affinity ligand activation of IgE receptors (Torigoe et al., 1998). This previous study of IgE receptor activation did not directly examine the level of Lyn in the antagonized receptors, however, leaving open the possibility that an inhibition of kinase function rather than sequestration was involved, which would be consistent with the Lck data described here.

Stotz et al. (1999) and Daniels et al. (1999) failed to see cross-antagonism using either a dual TCR hybridoma or short-term line with two TCR. Although these two prior studies used $CD8^+$ T cells with class I ligands, we do not think that this accounts for the different findings, as

we have seen similar accelerated recruitment of SHP-1 to the TCR using antagonists with human $CD8^+$ T cells (Stefanova et al., unpublished data). Thus, the divergent outcomes may indicate that multiple mechanisms, including both active negative regulation and competition for TCR engagement, can contribute to antagonist function. However, SHP-1 infection of TCR other than those directly engaged by antagonist is likely to be less extensive than SHP-1 recruitment to the engaged TCR themselves. In this case, antagonist recognition followed by dissociation and then agonist binding to the same TCR pool would result in visible inhibition due to the high level of SHP-1 preloading. In contrast, agonist binding to a distinct set of receptors might result in a much more modest, perhaps unmeasurable, effect if infectious spread of SHP-1 among receptors of distinct specificity were more limited. This could lead to a failure to see transinhibition in a two TCR system, especially using optimal agonists, while still allowing antagonists engaging the same TCR pool as the agonist to cause inhibition through SHP-1 recruitment. The description here of a molecular phenotype for TCR affected by SHP-1 negative feedback provides a method to directly investigate this possibility.

T cell activation has recently been shown to be hierarchical in nature, both on a kinetic and quantitative basis. Thus, different responses require a shorter or longer duration or a greater or lesser amount of effective TCR signaling to reach the threshold necessary for their elicitation (Viola and Lanzavecchia, 1996; Itoh and Germain, 1997; Iezzi et al., 1998). Analysis in parallel of multiple variant ligands for the same TCR has demonstrated that a higher density of partial agonist ligand is needed to reach the same total generation of effective intracellular signals as agonist but that when such similar total signaling occurs the responses induced by the two distinct ligands for many (though not all) measurable parameters are the same (Hemmer et al., 1998). Thus, T cells for the most part have their activity directed by the integrated level of effective signaling, rather than by the presence of unique signals induced by partial agonists/antagonists. In the context of the results described here, this suggests that TCR antagonists mediate their inhibitory effects by limiting effective signaling in either amount and/or duration. Our previous studies have shown that like antagonists, agonists induce SHP-1 association with the TCR and interfere with Lck function, but they do so with a substantial time delay. Antagonists alter the rate of such TCR desensitization, reducing the time over which complete signals are delivered and also limiting the absolute amount of effective signal at any time to a level below the threshold for certain responses. This should result in more effective inhibition of those outcomes requiring the most/longest duration of TCR signaling, and this is seen in experiments analyzing antagonist inhibition of more than a single response parameter (Hemmer et al., 1998). Viewed in this manner, TCR antagonism is primarily a disturbance in the regulation of TCR desensitization that typically serves to shut down an effective signaling response after sufficient time has passed for the proper gene activation events to transpire.

Experimental Procedures

Mice

B10.PL ($I\text{-}A^u$) and B10.BR ($I\text{-}A^k$) mice were purchased from the Jackson Laboratory. The MBP TCR transgenic (tg) mouse and the D10 TCR tg mouse were generated as previously described (Hardardottir et al., 1995; Dittel et al., 1997, respectively). (B10.BR/B10.PL)F1 mice and D10/MBP double tg mice were generated by intercrossing and maintained in our colony at Yale University.

Peptides and Antibodies

The MBP Ac1-11 and the MBP analog peptides K4A, K4M, Q3A, Q3AK4A, Q3AK4M, R5L, K4AR5L, and K4MR5L and the CA134-146 and the CA134-146 analog peptides W7M and E8A (Dittel et al., 1997) were synthesized at Yale University. All analog peptides are named using the single letter code for the residue found in the native protein at the position given with a number, followed by the single letter code for the residue to which it was changed. Thus, a peptide with the lysine (K) at position 4 of Ac1-11 substituted with an alanine (A) is called K4A. The following antibodies were used in these experiments: 3D3 (clonotypic mAb specific for the D10 TCR) (Kaye et al., 1983); 19G (clonotypic mAb specific for the MBP TCR) (Baron et al., 1993); goat anti-mouse-FITC (Fc specific) (Sigma Chemical); streptavidin-PE (Caltag); 2.4-G2 (anti-Fc receptor) (American Type Culture Collection, Rockville, MD); rabbit antiserum to ZAP-70, ζ Lck (Burkhardt et al., 1994), and SHP-1 (Upstate Biotechnology); 4G10 (anti-phosphotyrosine [Upstate Biotechnology]; H57 (anti-mouse TCRβ [PharMingen]); and peroxidase-linked goat antibodies to mouse and rabbit IgG (Bio-Rad Laboratories).

T Cells

The D10 TCR Th2.3 and D10.2.2 clones were generated from a D10 tg mouse, the MBP.31 clone was generated from a MBP tg mouse, and the D10/MBP 5-3 clone was generated from a D10/MBP dual TCR tg mouse as previously described (Dittel et al., 1997). The clone 19 clone has been previously described (Baron et al., 1993).

Immunofluorescence

Immunofluorescence staining using anti-D10 TCR (3D3-biotin detected with SA-PE) and anti-MBP TCR (19G detected with anti-mouse FITC) was carried out as previously described (Dittel et al., 1997) on clones generated from the D10 tg, MBP tg, and the D10/MBP tg mice.

Proliferation and Antagonist Assays

T cell proliferation and antagonist assays were carried out as previously described (Dittel et al., 1997). In brief, $1–2 \times 10^4$ T cells were incubated with $1–2 \times 10^5$ splenocytes from B10.BR, B10.PL, or (B10.BR/B10.PL)F1 mice in the presence of peptides. For antagonist assays, the splenocytes were prepulsed with agonist CA134-146, K4A, or K4M peptides for 2 hr at 37°C in serum free medium. MAb inhibition of proliferation was performed using 1×10^4 D10/MBP 5-3 cells cocultured with 1×10^5 splenocytes from B10.PL or B10.BR mice in the presence of 0.5 μg/ml Ac1-11 or 0.05 μg/ml CA134-146, respectively. MAb 2.4-G2 was added (10 μg/ml) to block Fc receptors on the APC. Either serial 1:10 dilutions of purified 3D3 were added from 0.001 to 10 μg/ml, or serial 1:2 dilutions of 19G ascites from 1:150 to 1:2400 were added. Cultures were pulsed after 48 hr with 0.5–1 μCi of [^{3}H]TdR and harvested after 15–18 hr.

Stimulation and Processing of T Cells for Analysis of Signaling Molecules

T cells (5×10^6) were added to peptide-pulsed L cells stably transfected with cDNA expression construct encoding $I\text{-}A^k$ or $I\text{-}A^u$ (2×10^6) or to splenocytes from (B10.BR/B10.PL)F1 mice (25×10^6). After centrifugation at 1000 rpm for 10 s, the cells were incubated at 37°C for the indicated period of time. After incubation, cells were washed once with PBS and placed in lysis buffer containing 1% NP-40 (ZAP-70 immunoprecipitation) or 1% Brij96 (TCR immunoprecipitation), 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, 1 mM Na_3VO_4 (Sigma), and complete protease inhibitor cocktail (Boehringer Mannheim). After removal of nuclear debris by

centrifugation, the resultant supernatants were subjected to immunoprecipitation and analyzed by immunoblotting or by an in vitro kinase assay.

For sequential stimulation, the I-A^u-expressing L cells were first incubated with magnetic beads covered with goat anti-mouse Ig (Dynal, Oslo, Norway) for 8 hr at 37°C. Cells containing beads were recovered by magnet, washed, and preincubated with antagonist peptide (Q3AK4M; 300 μg/ml) for 6 hr at 37°C. After this period, T cells were added and incubated in the continued presence of Q3AK4M peptide for 6 hr. Control T cells were exposed for the same time period to I-A^u cells containing magnetic beads but in the absence of antagonist. Bead-containing cells were removed by magnet, and the T cells were washed. For signaling studies, control and antagonist preexposed T cells (1×10^6) were added to agonist (CA136-147, 100 μg/ml)-pulsed I-A^k presenting cells (1×10^6), spun at 1000 rpm for 10 s, and incubated at 37°C for 5 min.

Immunoprecipitation, Protein Kinase Assay, and Immunoblotting

ZAP-70 and TCR were immunoprecipitated by incubation of lysates with optimized amounts of polyclonal antisera or mAb on ice for 4 hr. Immunocomplex protein kinase assays, SDS-PAGE, and immunoblotting were performed as previously described (Stefanova et al., 1993). For the protein kinase assay, immunoprecipitates were incubated with 50 μl of 25 mM Hepes (pH 7.2), containing 3 mM $MnCl_2$, Brij96 (0.1%), and 1 μCi of [γ-^{32}P]ATP (NEN). After incubation for 10 min at 25°C, proteins were eluted in sample buffer and subjected to SDS-PAGE and autoradiography. Quantitative data were obtained from film exposures using a Molecular Dynamics Laser Densitometer.

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