# Dimerization of Soluble Major Histocompatibility Complex–Peptide Complexes Is Sufficient for Activation of T Cell Hybridoma and Induction of Unresponsiveness

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## Summary

Major histocompatibility complex (MHC) class I molecules are cell-surface proteins that present peptides to $CD8^+$ T cells. These peptides are mostly derived from endogenously synthesized protein. Recombinant, soluble MHC class I molecules were produced, purified, and loaded homogeneously with synthetic peptide. These MHC–peptide complexes were used to activate a T cell hybridoma. While monomers of MHC–peptide bound to the T cell, they showed no stimulatory activity. Dimers fully triggered the T cell hybridoma to secrete interleukin 2. This response was followed by a state in which the T cell was refractory to restimulation as a result of defective signal transduction through the T cell receptor.

T lymphocytes recognize through TCR a molecular complex consisting of an antigenic peptide bound to an MHC-encoded molecule on the surface of APC (1). In developing thymocytes, TCR engagement can either induce cell death or rescue from a programmed cell death (2). Tolerance to self and self-MHC restriction are thought to be in large part the result of these interactions through processes referred to as thymic selection and education. In mature T cells, TCR engagement can either induce clonal expansion and differentiation or lead to a long-lived state of unresponsiveness called anergy (3). In Th1 $CD4^+$ cells, antigen receptor occupancy (signal 1) in conjunction with a second costimulatory signal synergizes to produce IL-2 and leads to T cell activation (4). In the same cells, TCR engagement alone in the absence of costimulation leads to anergy or deletion. Less is known about $CD8^+$ T cells. In a few instances, anergy can be induced using fixed APC or immobilized anti-TCR mAb, but such anergized $CD8^+$ T cells maintained CTL killing activity (5, 6). Anergy has been proposed to explain the immune tolerance to self-antigens not presented in the thymus by a process referred to as peripheral tolerance (7).

In vitro models have been developed that have allowed the identification of some of the surface molecules through which the costimulatory signals (signal 2) are delivered. A dominant role has been demonstrated in mouse $CD4^+$ T cells for CD28 (8). Coexpression on the same APC of the B7 molecule (the ligand of CD28) and a specific MHC–peptide complex allows the induction of $CD8^+$ CTL (9). Other cell surface molecules like CD2, leukocyte function–associated antigen-1, or very late antigen-4 have also been implicated (10). After antigenic stimulation, T hybridoma cells become unresponsive to restimulation (11, 12). This state, which lasts for 2 to 3 wk, resembles anergy as defined by functional inactivation and failure to produce IL-2 (13).

The ability to produce recombinant MHC molecules and to load them with exogenous peptides provides a more quantitative approach to addressing this issue and studying T cell triggering. We have previously constructed a single-chain, soluble MHC class I H-$2K^d$ molecule (SC-$K^d$)[1] by connecting the COOH terminus of the ectodomain of the native H-$2K^d$ molecule to the $NH_2$ terminus of the mouse $\beta_2$-microglobulin through a 15–amino acid–long spacer (14). Unlike native MHC molecules purified from cells, this recombinant molecule is soluble in the absence of detergent and is homogeneous with respect to the $\beta_2$-microglobulin, containing only $\beta_2$-microglobulin of mouse origin. It was produced in large quantities using baculo virus–infected insect cells, yeast, or Chinese hamster ovary cells and was shown to bind synthetic peptides with the same specificity as the native, cell surface–associated H-$2K^d$ (15–17).

In the present study, we show that recombinant single-chain SC-$K^d$ molecule loaded with an antigenic peptide is

[1] *Abbreviations used in this paper:* PKC, protein kinase C; SC-$K^d$, single-chain soluble MHC class I H-$2K^d$ molecule.

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capable of binding to its cognate TCR and activating a T cell hybridoma or a basophilic cell transfected with the TCR fused to CD3-$\zeta$. Cell activation requires at least dimerization of the MHC molecules. In T hybridoma cells, as it has been shown using antigen-bearing APC (11, 12), this activation is followed by a state of unresponsiveness, which results from a block in signal transduction through the TCR.

## Materials and Methods

*Peptides.* The Cw3 (RYLKNGKETL), NP (TYQRTRALV), and PbCS (YIPSAEKI) peptides have been previously described (18) and were obtained after HPLC purification from Neosystem SA (Strasbourg, France).

*mAbs.* The SF1-1.1.1 mAb (HB158; American Type Culture Collection, Rockville, MD) is specific for the $\alpha$3 domain of $K^d$ (17). The 20-8-4S recognizes an epitope in the $\alpha$1-$\alpha$2 domains of $K^d$ or $K^b$ (19). These two mAb were purified on protein A–Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) as described (20). The H57-597, which recognizes the C$\beta$ domain of the TCR (21), was purchased from Pharmingen (San Diego, CA). The F23.1 mAb (22), which recognizes a determinant on V$\beta$8$^+$ TCR, was generously provided by P. Marche (Pasteur Institute, Paris, France). Fab and $F(ab')_2$ fragments were prepared as described (20).

*Preparation of Soluble $K^d$.* The SC-$K^d$ sequence (14) was cloned into the amplifiable expression vector pFRSV (23) and transfected into Chinese hamster ovary cells. Clones resistant to 0.5 $\mu$M methotrexate were selected and subjected to stepwise increases in methotrexate concentration from 1 to 500 $\mu$M. Secreted $K^d$ was monitored in the culture supernatant by ELISA in microtitration plates coated with SF1-1.1.1 and revealed using biotinylated 20-8-4S and horseradish peroxidase–labeled streptavidin (Sigma Chemical Co., St. Louis, MO). High-producer transfectant clones were cultivated in roller bottle in $\alpha$-MEM supplemented with 2% FCS. SC-$K^d$ molecules were purified by affinity chromatography on protein A–Sepharose coupled with the SF1-1.1.1 mAb. Before use, SC-$K^d$ preparations were ultracentrifuged for 1 h at 200,000 *g* to remove any aggregate.

SC-$K^d$ molecules were loaded with the antigenic peptide and dimerized using the SF1-1.1.1 mAb. In some experiments, dimers were purified by high-performance gel filtration on Superose 6 HR (Pharmacia LKB Biotechnology, Inc.) using a flow rate of 0.35 ml/min of PBS.

*T Cell Activation.* The T cell hybridoma 9.4, previously (24) obtained by fusion between the 58$\alpha^-/\beta^-$ T cell thymoma (25) and the CW3/1.1 CTL clone (26), is specific for the Cw3 peptide presented by H-2$K^d$. T cells ($10^5$) were cultured for 24 h in 200 $\mu$l of RPMI 1640 medium supplemented with 10% FCS containing the indicated $K^d$–peptide and mAb or were cultured in microtitration plates that had been coated with $K^d$–peptide by overnight incubation in PBS. Culture supernatants were collected and their IL-2 content was assayed by measuring the effect of serial dilutions on CTLL-2 (American Type Culture Collection T1B 214) proliferation in presence of [$^3$H]thymidine (New England Nuclear, Boston, MA). In some experiments, T cells were reisolated, washed, and allowed to grow for 3 d at 2 × $10^5$/ml in fresh RPMI 1640 containing 10% FCS. Reactivation was assayed as previously or in the presence of 10 ng/ml PMA and 250 ng/ml $Ca^{2+}$ ionophore A23187 (Sigma Chemical Co.).

*Basophil Transfection.* The V$\alpha$-C$\alpha$ and V$\beta$-C$\beta$ segments of the Cw3/1.1 TCR were fused to transmembrane and cytoplasmic domains of the $\zeta$ chain by PCR (27). These constructs were cotransfected into RBL-2H3 cells by electroporation using the pAG60 plasmid as marker (28). Transfectants were selected in DMEM supplemented with 10% FCS and 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD). High expressers were selected by flow cytometry using the H57-597 mAb. 2 wk before cell activation, the G418 was removed from the culture medium. Cells were labeled for 15 h with serotonin binoxalate (1,2-$^3$H(N)), washed, and incubated for 1 h with dimers of $K^d$–peptide. Serotonin exocytosis was measured by counting the radioactivity released in the culture medium as previously described (29).

## Results

Single-chain SC-$K^d$ loaded with peptide activates basophils transfected with a TCR-$\zeta$ fusion. We previously showed that single-chain, soluble SC-$K^d$ binds peptide (16). We wanted to determine if it could activate a cell bearing a $K^d$-restricted TCR. The T cell chosen for this study (Cw3/1.1) was previously obtained by immunizing DBA/2 mice with P815 transfected with the HLA-Cw3 gene (30). Cw3/1.1 is $K^d$ restricted and specific for a 10-mer peptide derived from HLA-Cw3 (hereafter referred to as the Cw3 peptide). Its TCR had been cloned and sequenced (31).

To test whether the single-chain SC-$K^d$ was biologically active, the $\alpha$ and $\beta$ chains of the Cw3/1.1 TCR were fused to the $\zeta$ chain of the CD3 complex, and the two fusion genes were transfected into the basophilic cell line RBL-2H3 (29). Transfected cells expressing high levels of the chimeric TCR were selected by flow cytometry using the anti-C$\beta$ H57 mAb. Cell activation was measured by incubating the basophilic transfectants with SC-$K^d$ and SF1-1.1.1 (an anti-$K^d$ mAb recognizing a single epitope in the $\alpha$3 domain) and quantitating serotonin exocytosis.

As shown in Fig. 1, a specific activation was observed with SC-$K^d$ loaded with Cw3, but not with unloaded SC-$K^d$ or with SC-$K^d$ loaded with an unrelated peptide (PbCS). In the absence of SF1-1.1.1, no cell activation was observed. This indicates that the single-chain SC-$K^d$ interacts with its cognate TCR in a peptide-specific manner and transduces an activation signal.

*Single-chain SC-$K^d$ Loaded with Peptide Activates a T Cell Hybridoma.* To test for the ability of the single-chain SC-$K^d$ to act as an antigen-presenting molecule to a T cell, purified SC-$K^d$ was charged with Cw3, coated onto a microtiter plate, and tested for the ability to stimulate the 9.4 T cell hybridoma. This T cell hybridoma was previously obtained by fusing the Cw3/1.1 T cell clone with the 58$^{\alpha-/\beta-}$ T cell hybridoma, which is devoid of endogenous TCR (24). Activation of the 9.4 hybridoma results in IL-2 secretion, which can be monitored by measuring the capacity of the culture supernatant to sustain IL-2–dependent CTLL-2 proliferation.

As shown in Fig. 2, when SC-$K^d$ was loaded with Cw3 and coated onto the culture plate, it activated the 9.4 hybridoma in a dose-dependent manner. This activation was peptide specific, as SC-$K^d$ loaded with an unrelated peptide (PbCS) had no activity.

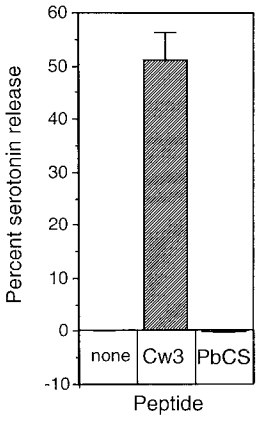


**Figure 1.** Basophil activation by recombinant SC-K$^d$–Cw3. Basophils transfected with the TCR-$\zeta$ fusion were incubated for 1 h with 25 $\mu$g of SC-K$^d$ loaded with the indicated peptide and dimerized with the SF1-1.1.1 mAb. Serotonin exocytosis was measured in the culture supernatant. Results are expressed as (experimental − spontaneous serotonin release)/(total − spontaneous serotonin release) × 100.

*To Activate T Cells, SC-K$^d$ Must Be at Least Dimeric.* In the experiment shown in Fig. 2, recombinant SC-K$^d$ was coated onto the plate, thereby mimicking the antigen-presenting cell surface. The availability of soluble monomeric MHC molecules allowed us to test for the requirement for multivalency for MHC molecules to trigger a T hybridoma cell.

Dimers of SC-K$^d$ loaded with Cw3 were prepared by incubating the MHC–peptide complexes with the half-stoichiometric amount of the SF1-1.1.1 mAb specific for a single epitope located in the $\alpha$3 domain of K$^d$ (17). This preparation was added to the T cell, and activation was measured by IL-2 secretion. As shown in Fig. 3, a dose-dependent stimulation was observed. Note that stimulating the 9.4 T cell hybridoma with dimeric SC-K$^d$ is more efficient than stimulating with coated monomers of SC-K$^d$, as higher secretion of IL-2 is observed at lower doses of SC-K$^d$ (compare Figs. 2 and 3). When a higher multivalency was induced by adding SC-K$^d$–Cw3, SF1-1.1.1, and an anti–mouse Ig antibody, T cell activation was observed at even lower concentrations of SC-K$^d$ (Fig. 4, line 6).

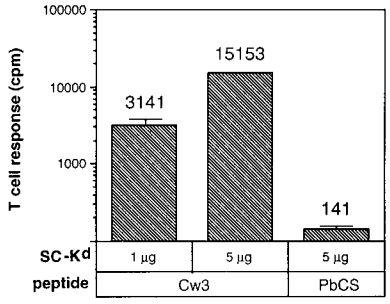


**Figure 2.** Activation of the 9.4 hybridoma by recombinant SC-K$^d$ coated on the plate. SC-K$^d$ was loaded with either the Cw3 peptide or with the unrelated peptide PbCS. The indicated quantities of loaded SC-K$^d$ were coated on the plate, and $10^5$ T cells were added. After 24 h at 37°C, IL-2 secretion in the supernatant was measured by its capacity to sustain the proliferation of the IL-2–dependent CTLL-2. Results are expressed as cpm of [$^3$H]thymidine incorporation by CTLL-2.

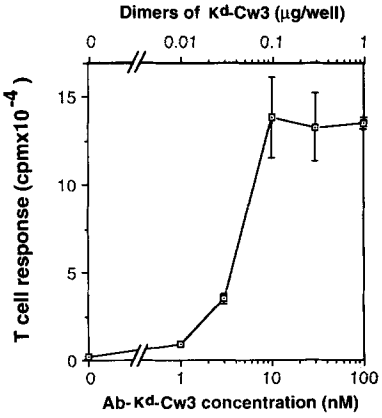


**Figure 3.** Activation of the 9.4 hybridoma by dimers of SC-K$^d$–Cw3. Purified SC-K$^d$ was loaded with Cw3 and dimerized using the SF1-1.1.1 mAb. This mixture was incubated at the indicated concentrations with $10^5$ T cells. After 24 h, culture supernatants were harvested and T cell response (IL-2 secretion) was measured as described in the legend of Fig. 2.

T cell activation was peptide specific, as dimers made of SC-K$^d$ molecules charged with unrelated peptides (PbCS or NP) did not stimulate the T cells (Fig. 4, *lines 4 and 5*). No stimulation was observed when the K$^d$-specific antibody was omitted (Fig. 4, *line 3*), when it was replaced by the 20-8-4S antibody (*line 7*) recognizing an epitope in the $\alpha$1-$\alpha$2 domain of K$^d$ (19), or when an unrelated antibody of the same isotype was used (*F23.1 in line 8*). Furthermore, when an excess of the anti-$\alpha$1-$\alpha$2 (20-8-4S) antibody was used in addition to the anti-$\alpha$3 (SF1-1.1.1) antibody, T cell stimulation was lost, whereas an excess of the unrelated antibody had no effect (Fig. 4, *lines 9 and 10*).

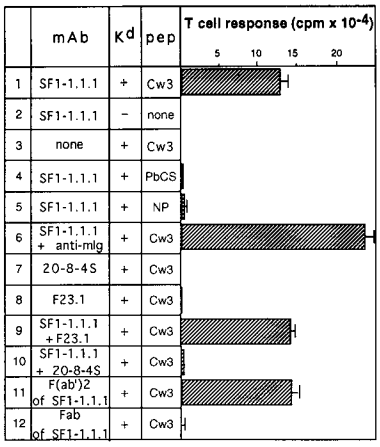


**Figure 4.** Activation of T cell hybridoma by SC-K$^d$–Cw3 requires dimerization through the SF1-1.1.1 mAb. SC-K$^d$ (0.3 $\mu$g) loaded with the indicated peptide was incubated with the indicated antibodies or antibody fragments. These mixtures were then incubated with $10^5$ T cells, and T cell activation was measured as described in the legend of Fig. 2. In line 6, complex aggregates of K$^d$–Cw3 were obtained using the SF1-1.1.1 mAb and an anti–mouse Ig antibody. In lines 9 and 10, the 20-8-4S mAb (anti-$\alpha$1-$\alpha$2) and the F23.1 (unrelated mAb of same isotype) were in a 10-fold molar excess over the SF1-1.1.1 mAb.

To further prove that, in our experimental system, dimerization is required for activation of T cell hybridoma, SC-$K^d$–Cw3 complexes were incubated with either Fab or $F(ab')_2$ fragments prepared from the SF1-1.1.1 mAb and were used to activate the 9.4 hybridoma. As shown in Fig. 4 (*lines 11 and 12*), while Fab fragments had no effect, $F(ab')_2$ were as efficient as native Ig in stimulating the T cell hybridoma.

Activation of the T cell hybridoma could be due to high-order aggregates rather than to truly bivalent complexes. To prove that dimers were active, we purified material consisting of one mAb molecule and two SC-$K^d$–Cw3 complexes by high-performance gel filtration. As shown in Fig. 5, such purified dimers running with an apparent molecular mass of 250 kD could activate the 9.4 hybridoma cell line.

In summary, in this system, monomers of MHC molecules do not activate T cells. Dimers and higher multimers give excellent responses. This activation is blocked by an antibody recognizing the apical portion ($\alpha1$-$\alpha2$) of the MHC molecule.

*Dimerization of MHC Molecules Is Required for Signal Transduction, Not for Binding.* Initial measurements indicate that TCR affinity for its ligand covers a wide range of values, with $K_D$ from $10^{-4}$ to $10^{-7}$ M (32–35). As the concentration of SC-$K^d$ used in our assay is $\sim 10^{-7}$ M, one could argue that the anti-$K^d$ antibody is required to stimulate the T cell hybridoma because dimerization increases the apparent avidity of the complex for the TCR. Accordingly, the antibody would be required for binding, not for triggering.

To rule out this interpretation, we designed the following experiment. We took advantage of the slow off-rate of the SF1-1.1.1 mAb. As a consequence of this off-rate, $K^d$–peptide complexes dissociate very slowly once they are bound to this mAb (16). SC-$K^d$ molecules were loaded with either the Cw3 peptide or the unrelated (nonstimulating) PbCS peptide. While keeping the concentration of SC-$K^d$–Cw3 constant, increasing concentrations of SC-$K^d$–PbCS and SF1-1.1.1 mAb were added to the 9.4 hybridoma cells. In one case, the SC-$K^d$–peptide complexes were first mixed with the SF1-1.1.1 mAb, and these preformed dimers were then added to the T hybridoma cells. In the other case, SC-$K^d$–peptide complexes were first incubated with the T hybridoma cells, and the SF1-1.1.1 mAb was added afterward.

As shown in Fig. 6, when the SC-$K^d$–peptide dimers were preformed (*open squares*), increasing the concentration of SC-$K^d$–PbCS led to a progressive loss of T cell activation. This probably reflected the formation of a decreasing number of dimers presenting two Cw3 peptides. In contrast, when the two SC-$K^d$–peptide complexes were first added to the hybridoma cells (*closed squares*), even a 30-fold excess of SC-$K^d$–PbCS over SC-$K^d$–Cw3 had no negative effect on T cell activation. This indicates that, in the absence of SF1-1.1.1, monomers of SC-$K^d$–Cw3 complexes interact with the T cell hybridoma cell.

*Activation of T Cell Hybridoma by MHC–Peptide Complexes Is Followed by a State of Unresponsiveness.* Antigen recognition can, under certain conditions, lead to functional inactivation. In preliminary experiments, T hybridoma cells were activated with P815 cells transfected with the HLA-Cw3 gene, reisolated, and retested for activation. As already reported for other T hybridomas (12), previously activated T cells became unresponsive (Abastado, J.-P., unpublished observations).

To test whether TCR engagement by purified MHC–peptide dimers could mimic this phenomenon, we performed the same experiments with SC-$K^d$ loaded with Cw3. T hybridoma cells were activated for 24 h using increasing concentrations of dimers of SC-$K^d$–Cw3. Cells were then washed, cultivated for 3 d, and retested for activation by SC-$K^d$–Cw3. As shown in Fig. 7, increasing the concentration of SC-$K^d$–Cw3 used for the first stimulation gradually reduced the response to a second stimulation. T cell activation by concentrations of SC-$K^d$–Cw3 above 500 nM induced a state of unresponsiveness in which cells were refractory to further activation.

To assay the specificity of this induction of unresponsiveness, cells were incubated with SC-$K^d$ loaded with either Cw3 or an unrelated peptide (PbCS). After reisolation as described above, T cell response was compared with the response of untreated cells (Fig. 8). Whereas cells activated by SC-$K^d$–Cw3 yielded only 3% of the primary response, cells in-

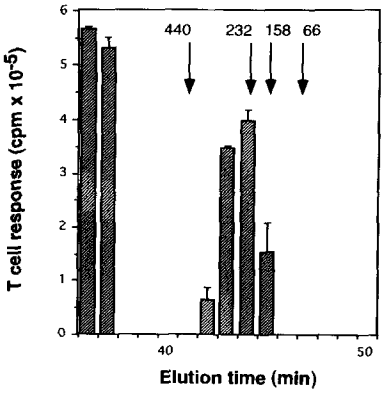


**Figure 5.** Purified dimers of SC-$K^d$–Cw3 activate the 9.4 T cell hybridoma. SC-$K^d$ (0.4 mg) was loaded with the Cw3 peptide and incubated with SF1-1.1.1 mAb (0.6 mg) for 1 h. This mixture (425 $\mu$l) was separated by high-performance gel filtration on a Superose 6 HR column using albumin (66 kD), aldolase (158 kD), catalase (232 kD), and ferritin (440 kD) as size markers. Fractions containing SC-$K^d$ were identified by ELISA. 50 $\mu$l of each fraction was incubated with $10^5$ T hybridoma cells. T cell activation was measured as described in the legend of Fig. 2.

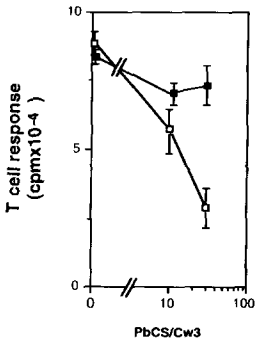


**Figure 6.** SC-$K^d$ dimerization is required for T cell stimulation, not for binding. A mixture of SC-$K^d$ loaded with Cw3 and SC-$K^d$ loaded with PbCS at the indicated ratio was incubated with SF1-1.1.1 for 2 h and then with the 9.4 cells (*open squares*). Alternatively, the mixture was incubated first with the 9.4 cells (3 h), and then the SF1-1.1.1 mAb was added (*closed squares*). T cell activation was measured as described in the legend of Fig. 2. Only when the dimers were preformed was SC-$K^d$–PbCS inhibitory.

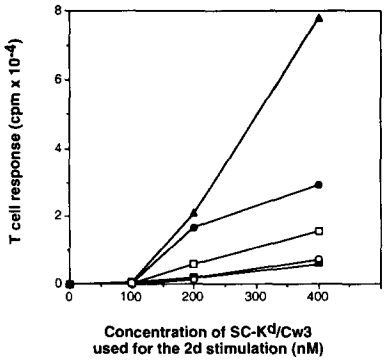


**Figure 7.** Upon activation by SC-K$^d$–Cw3, T cell hybridoma become refractory to subsequent stimulation. T cells (5 × $10^5$/ml) were incubated for 24 h with dimers of SC-K$^d$–Cw3 at the following concentrations: 2,000 nM (–■–), 1,000 nM (–○–), 500 nM (–□–), 250 nM (–●–), and 0 nM (–▲–). Cells were then reisolated, grown in complete medium for 3 d, and retested for activation by dimers of SC-K$^d$–Cw3 at the concentration indicated on the x-axis of the figure. T cell activation was measured as described in the legend of Fig. 2.

cubated with SC-K$^d$–PbCS or PBS gave a response identical to the primary response (Fig. 8).

*Unresponsiveness Does Not Result from Selection of Preexisting Cell Variants.* For T cell hybridomas, TCR occupancy can result in reduced cell proliferation (36) and activation-driven cell death (37). The above described unresponsiveness could therefore result from selection of previously existing response variants unable to respond to antigen. To test out this hypothesis, we measured cell viability by trypan blue exclusion and apoptosis induction by propidium iodide staining and flow cytometry (38). No significant difference could be detected between cells incubated with dimers of SC-K$^d$–Cw3 or dimers of SC-K$^d$–PbCS (data not shown). We also measured the ability of 9.4 cells to form colonies at limiting dilution in microtiter plates coated either with dimers of SC-K$^d$–Cw3 or with dimers of SC-K$^d$–PbCS. Although cell colonies grew with a slight delay in wells coated with the antigenic complex (SC-K$^d$–Cw3), plating efficiencies were similar under both conditions (49 and 59%, respectively). Finally, we reisolated T cells at different time points after the first antigenic stimulation so that we could control cell expansion. Even under conditions where no cell expansion was observed, T cells that had been previously exposed to dimers of SC-K$^d$–Cw3 gave a reduced IL-2 secretion (Fig. 9). Taken together, these observations indicate that SC-K$^d$–Cw3 dimers do not induce massive cell death and that unresponsiveness does not result from selection of preexisting unresponsive cells.

*Unresponsiveness Induced by SC-K$^d$–Cw3 Does Not Result from Reduced TCR Expression but from Defective Signal Transduction.* Unresponsiveness induced by SC-K$^d$–Cw3 could result from TCR down-modulation. Cells were treated with dimers of SC-K$^d$ loaded with Cw3 or an unrelated peptide (PbCS) and were reisolated as described above. TCR expression was measured by flow cytometry on reisolated populations. As shown in Fig. 10, no difference was observed in cell surface expression of the TCR. Untreated cells, cells incubated with SC-K$^d$–PbCS, and cells incubated with PBS were indistinguishable (not shown).

The same T cell populations were compared for activation by dimers of SC-K$^d$–Cw3 or a combination of PMA and calcium ionophore A23187. These pharmacological agents are known to directly activate protein kinase C (PKC), which is involved in T cell activation. As shown in Fig. 8, cells treated with SC-K$^d$–Cw3 (activated) were refractory to a second stimulation by SC-K$^d$–Cw3 but gave the same response to PMA and calcium ionophore as untreated cells or cells incubated previously with SC-K$^d$–PbCS (control). This indicates that T cell response could be restored by direct stimulation of the PKC.

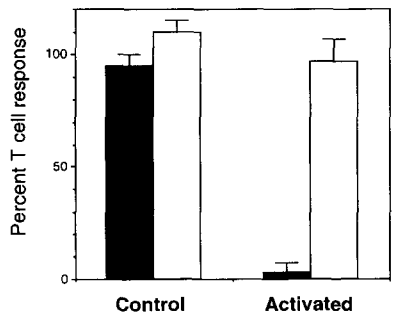


**Figure 8.** Induction of unresponsiveness is peptide specific and is due to deficient signal transduction. T cells ($10^5$) were incubated with 20 μg of SC-K$^d$ loaded with either Cw3 (activated) or PbCS (control) for 24 h in 200 μl of medium. Cells were then reisolated, grown in complete medium for 3 d, and retested for activation by 4 μg SC-K$^d$ loaded with Cw3 (*closed bars*) or by PMA and ionophore A23187 (*open bars*). T cell activation was measured as described in the legend of Fig. 2. Results are expressed as percentage of the primary response.

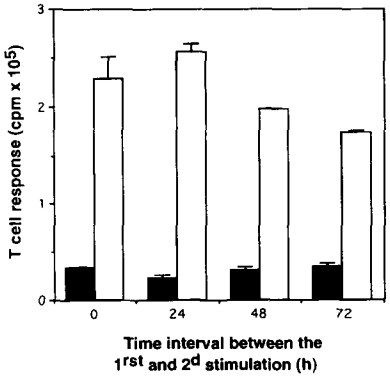


**Figure 9.** Induction of unresponsiveness does not require cell amplification. T cells ($10^5$) were incubated for 24 h with 10 μg of SC-K$^d$–Cw3 (*closed bars*) or SC-K$^d$–PbCS (*open bars*). Cells were then reisolated, incubated in fresh culture medium for the indicated period of time, and retested for activation by 1 μg of SC-K$^d$–Cw3. Secondary T cell response was measured as described in the legend of Fig. 2.

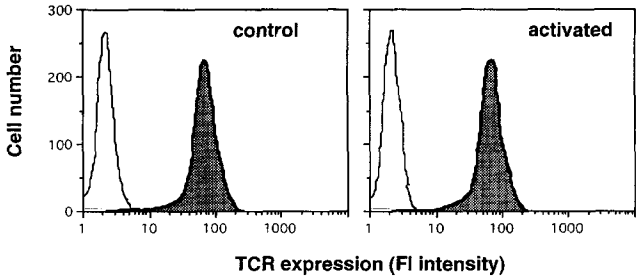


**Figure 10.** Induction of unresponsiveness does not result from reduced TCR expression. T cells ($10^5$) were incubated with 20 $\mu$g of SC-$K^d$ loaded with Cw3 (activated) or PbCS (control) for 24 h in 200 $\mu$l of culture medium. Cells were then reisolated, grown in complete medium for 3 d, and tested for TCR expression by flow cytometry using the anti-C$\beta$ H57-597 mAb.

## Discussion

In a previous study, we demonstrated that SC-$K^d$ can bind antigenic peptides with the same specificity as the native $K^d$ molecule (17). Here we show that this recombinant molecule is fully functional and can act as an antigen-presenting molecule to stimulate a T cell hybridoma and to induce a state of unresponsiveness. Monovalent SC-$K^d$ molecules show no activity, but dimerization is sufficient to make them active.

The first experimental system used to prove the functionality of the SC-$K^d$ molecule is a basophil expressing a chimeric TCR composed of the extracellular domains of the $\alpha$ and $\beta$ chains fused to the transmembrane and intracellular domains of the $\zeta$ chain of the CD3. This system, developed by Engel et al. (29), was chosen because it is very sensitive and can detect low-affinity interactions. The peptide specificity of the basophil activation and the fact that TCR interaction occurs with the apical portion of the MHC molecule suggest that the observed MHC–peptide interaction with the chimeric TCR is similar to the physiological interaction between a native MHC molecule expressed on an APC and the native TCR expressed on a T cell.

To further prove the functionality of our recombinant MHC molecule in a less artificial system, SC-$K^d$–peptide was used to activate a T cell hybridoma. Again, a specific activation was obtained when SC-$K^d$ was coated on a plate or when it was dimerized using an anti-$\alpha$3 mAb or F(ab')$_2$ fragments of this mAb. The latter observation shows that the Fc region of the cross-linking mAb does not play any role in the activation process. Monovalent SC-$K^d$–peptide, even in the presence of Fab fragments of the anti-$\alpha$3 mAb, failed to induce IL-2 secretion, although we cannot rule out the possibility that they nevertheless generated some second messenger signal. Activation by dimers could be blocked by an anti-$\alpha$1-$\alpha$2 mAb, again supporting a role for this portion of the MHC molecule in the interaction with the TCR.

Another soluble recombinant MHC molecule had been previously used to activate T cells (39, 40). However, the minimal level of multivalency required for activation had not been addressed. Here we show that dimerization is sufficient but that a higher multivalency level makes activation more efficient. For many cell surface receptors, dimerization is sufficient to signal cellular responses. This has been extensively studied in the case of the epidermal growth factor receptor, the human growth hormone receptor, and the high-affinity receptor for IgE ($Fc_\epsilon RI$) (for review see reference 41). Comparison with the latter system may be particularly relevant to the present study, as the Fc receptor's $\gamma$ chain is related to the $\zeta$ chain of CD3 complex of the TCR, and signal transduction mechanisms are likely to be similar in the two systems.

The level of multivalency required for T cell activation is a highly debated question. Some authors (42–45) argue that dimers are insufficient for effective intracellular signaling, whereas others (46–49) claim that anti-TCR mAb Fab fragments can trigger T cells. In the present study, we find that dimers of MHC–peptide complexes are fully functional, even if higher-order aggregates are more efficient than dimers. One explanation for this finding could be that our MHC molecule preparations are contaminated with traces of aggregates. This appears unlikely, as (*a*) SC-$K^d$ preparations were ultracentrifuged before use; (*b*) in the absence of cross-linking antibody, SC-$K^d$–Cw3 complexes fail to activate basophil transfectants, a system in which dimers are known to be sufficient (41); and (*c*) dimers purified by high-performance gel filtration could activate T hybridoma cells. While each T cell may have different requirements, depending, for example, on the strength of the interaction of the TCR for its ligand, an important difference in our study is the type of stimulus used to trigger the T cell. H. Dintzis and coworkers (42–45) used a system in which the T cell response was not MHC restricted. Other studies used an anti-TCR mAb. We are using purified MHC–peptide complexes, a more physiological ligand. In general, much of what has been learned about T cell activation stems from studies in which the T cell stimulus was not its natural ligand. As shown in two recent studies (50, 51), a TCR can interpret subtle changes in its ligand, resulting in different tyrosine phosphorylation patterns and different signal transduction. Therefore, it might be of interest to reevaluate with recombinant MHC molecules some of the conclusions drawn from these previous experiments.

Why is multivalency required? Several explanations have been put forward (34, 39). The first states that multivalency is required for binding, as it enhances the apparent avidity of the MHC–peptide interaction with the TCR. The second proposes that multivalency is required for the stimulation itself. The experiment shown in Fig. 6 clearly shows that monomers of $K^d$–Cw3 bind to the Cw3-specific T cell hybridoma. This conclusion stems from the observation that when SC-$K^d$–Cw3 and SC-$K^d$–PbCS monomers are first incubated with the T cells and SF1-1.1.1 mAb is added subsequently, T cells respond by secreting IL-2. Even a 30-fold excess of SC-$K^d$–PbCS does not block this response. To explain this, we propose that T cells concentrate SC-$K^d$–Cw3 monomers by specifically binding them. Therefore, the concentration of SC-$K^d$–Cw3 on the T cell surface is always

high compared with the concentration of SC-$K^d$–PbCS, which is diluted into the medium. Once an mAb molecule has bound a SC-$K^d$–Cw3 complex, it is more likely to bind another SC-$K^d$–Cw3 rather than a SC-$K^d$–PbCS. Addition of SF1-1.1.1 therefore dimerizes the TCR and triggers signal transduction. SC-$K^d$–Cw3 binding alone (in the absence of SF1-1.1.1 addition), however, is not sufficient to trigger the T cell. One possible (but not exclusive) explanation is that the interaction of SC-$K^d$–Cw3 with the TCR does not last long enough. By studying the kinetics of reaction between an alloreactive TCR and an MHC class I–peptide complex, two groups, using different approaches, have come to a similar conclusion, namely that the dissociation occurs at a rate that is too high (with a half-life for the complex of 27 s) to trigger the TCR (34, 35). Antibody addition could be required to decrease the effective off-rate. Alternatively, TCR dimerization could be needed for signal transduction.

Our study emphasizes the importance of TCR aggregation in T cell activation. Numerous in vitro studies using anti-TCR antibodies as the T cell stimulus indicate that TCR cross-linking is necessary for T cell triggering (52). It is not clear, however, if and how TCR aggregation occurs in more physiological circumstances, in particular when the stimulus is a class I MHC–peptide complex expressed at the surface of an APC. The fact that thousands of different peptides are presented by the same APC (53) seems to preclude the formation of extensive and stable patches of cross-linked class I MHC–peptide complexes. One crucial problem with multimeric MHC molecules is the question of whether all molecules in the multimer present the same peptide: If MHC molecules are already multimeric before the interaction with the TCR, the probability that all MHC molecules in the multimer are charged with the same peptide decreases rapidly with the size of the complex. The valence of MHC class I molecules expressed at the cell surface has been the subject of many investigations and debates. MHC class II molecules crystallize as dimers. For class I, it has been suggested that they are expressed at the cell surface as tetramers (54). However, these data were later reinterpreted (55). On the other hand, if monomers of MHC–peptide interact with monomers of TCR, what drives the aggregation of these complexes? T cell triggering has been shown to be a multistep process (56). Our results are compatible with a model in which MHC molecules interact with their cognate TCR as monomers. This interaction then drives the aggregation of the MHC–peptide–TCR complexes. An alternative hypothesis, which does not involve TCR aggregation, is that TCR engagement by MHC–peptide complexes induces a conformational change in TCR, which in turn transmits the signal inside the cell (57). This is not easily compatible with our data, unless TCR dimerization induces a conformational change in the CD3 complex.

Tolerance induction and maintenance are, in part, the results of clonal deletion of self-reactive cells in the thymus. Mature T cells can also be rendered anergic in the periphery by encountering antigen on nonprofessional APC. Here we show that purified dimers of MHC–peptide complexes induce a state in which the T hybridoma cell becomes unresponsive to a subsequent antigenic stimulation. This result confirms and extends previous observations made about transformed or normal T cells using either APC or anti-TCR mAb as stimuli (12, 58). This unresponsiveness is peptide specific, since SC-$K^d$ loaded with an irrelevant peptide has no effect. It does not result from reduced TCR expression (Fig. 10), as has been previously described (59). It is unlikely to be due to TCR blockade (60) by residual MHC–peptide complexes, as hyporesponsiveness is assayed after extensive T cell washing and 2 d after the first stimulus, a period of time during which T cells proliferate widely. Signal transduction through the TCR induces tyrosine kinases and phospholipase C$\gamma$1, which in turn leads to a rise in intracellular calcium and activation of PKC. PKC activation triggers a series of intracellular events, including AP-1 mobilization, which transactivates the IL-2 promoter. Here we showed that unresponsive T hybridoma cells have nevertheless conserved their capacity to produce IL-2, as IL-2 secretion can be restored when the cells are stimulated with phorbol ester and calcium ionophore, which are known to activate PKC directly. This suggests that unresponsiveness is due to defective signaling through the TCR and that this defect can be bypassed by directly activating PKC.

The induction of unresponsiveness by soluble recombinant MHC class I, as observed in this study, may help to evaluate the role of soluble MHC class I molecules in the induction of tolerance. Soluble MHC class I molecules circulate in the blood (61) as a result of shedding (62). Nonclassical class Ib molecules such as Qa-2 (63), Qb-1 (64), or Q10 (65) are actively secreted. The role of these soluble MHC molecules is still a matter of speculation. In transgenic mice expressing soluble forms of classical MHC class I molecules, some T cells were tolerized and others were not (66, 67). Our finding that monomers show no activity but that multimers induce T cell triggering followed by a state of unresponsiveness is consistent with a biological role for soluble MHC molecules produced by shedding.

The soluble, single-chain $K^d$ molecule described herein is fully functional when presented as dimers. Aggregates of dimers (as shown in Fig. 4) were even more efficient than dimers, suggesting that dimers, shown here to be necessary and sufficient for activation of hybridomas, may not be optimal. Reconstitution experiments as described in this study may prove useful to dissecting and evaluating, in a well-controlled experimental system, the requirements for T cell activation and induction of anergy. This could be of particular interest for finding new therapeutic approaches involving the specific modulation of immune responses.

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