The Magnitude of TCR Engagement Is a Critical Predictor of T Cell Anergy or Activation[1]

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Fast dissociation rate of peptide-MHC complexes from TCR has commonly been accepted to cause T cell anergy. In this study, we present evidence that peptides that form transient complexes with HLA-DR1 induce anergy in T cell clones in vitro and specific memory T cells in vivo. We demonstrate that similar to the low densities of long-lived agonist peptide-MHC, short-lived peptide-MHC ligands induce anergy by engagement of ~1000 TCR and activation of a similar pattern of intracellular signaling events. These data strongly suggest that short-lived peptides induce anergy by presentation of low densities of peptide-MHC complexes. Moreover, they suggest that the traditional antagonist peptides might also trigger anergy by a similar molecular mechanism. The use of short-lived peptides to induce T cells anergy is a potential strategy for the prevention or treatment of autoimmune diseases. ***The Journal of Immunology*****, 2004, 172: 5346–5355.**

Induction of tolerance in self-reactive memory T cells is an important process in the prevention and treatment of autoimmune diseases. Altered peptide ligands (APLs)[3] have been extensively studied as a potential means for induction of tolerance. APLs most commonly carry mutations at their TCR contact residues, creating different topologies of peptide-MHC at the TCR interface (1–3). As such, induction of anergy by APLs is commonly explained by the kinetic proofreading theory proposing that rapid TCR dissociation from a peptide-MHC ligand (4–6) leads to incomplete phosphorylation of signaling cascades directly linked to the TCR-CD3 complex. In contrast, we have recently reported, in several antigenic systems, $HA_{308-318}$/HLA-DR1, $HA_{109-120}$/I-E^d, OVA/I-A^d, that low densities of agonist peptide-MHC complexes cause anergy in the $CD4^+$ T cell clones (7) and in peripheral memory $CD4^+$ T cells (8). We suggested that low-avidity engagement of T cell receptors by low densities of agonist peptide might lead to the transduction of signals that cause T cell anergy. Hence, low numbers of peptide-MHC complexes engage fewer T cell receptors.

It is established, in several systems, that the binding of peptide ligands to MHC class II involves kinetic and structural intermediates (9–13). The intermediate peptide-MHC complex forms rapidly, has a flexible conformation, and is short-lived. The short-lived intermediate complex may slowly transform to a stable and rigid complex (11). Short-lived peptide-MHC complexes are important in the initiation of stable peptide binding, stabilization of empty MHC molecules, and in rescuing class II from denaturation at physiological temperatures (11). Additionally, rapid formation and dissociation of short-lived complexes yield peptide-receptive class II molecules that can readily bind peptides (14, 15). However, little is known about the role of short-lived peptide-MHC complexes in interaction with TCRs. Indeed, the prevalent view is that the class II Ag-processing pathway has evolved to select for long-lived peptides and that the formation of short-lived peptide-MHC complexes has no bearing on T cell stimulation.

We entertained the idea that short-lived variants of agonist peptides may deliver negative signals to T cells specific for the agonist peptide-MHC complexes. Because of the transient nature of the short-lived peptide-MHC complexes and Because of the structural flexibility that the peptide retains in the absence of a large anchor residue, one might predict that short-lived peptide analogues would present low net densities of the correctly positioned TCR contact residues interacting with the TCR (16).

In this study, by using variants of the $HA_{306-318}$ that are identical at the TCR-contacting residues but have altered MHC class II anchor residues to allow for transient (short-lived) binding to HLA-DR1, we show that anergy can be induced in $HA_{306-318}$-specific T cell clones in vitro and in vivo. Our findings demonstrate that short-lived peptide-MHC complexes and low doses of long-lived agonist peptide appear to induce anergy by similar mechanisms, supporting the notion that short-lived peptide-MHC complexes play important regulatory roles in T cell activation. The use of short-lived peptides to induce T cell anergy in an Ag-specific manner may be a potential strategy for the prevention or treatment of autoimmune diseases.

Materials and Methods

Cells and mice

Clone 1 (CL-1) is a $CD4^+$ Th1 clone specific for the influenza hemagglutinin (HA)-derived peptide $HA_{306-318}$ bound to HLA-DR1 (1, 17). EBV 1.24 transformed, a human HLA-DR1 (DRB1*0101)-positive B cell line was used as the APC for CL-1 stimulation. T cell clones and B cells were grown as described previously (7). IL-2-sensitive cell lines, HT-2 (American Type Culture Collection, Manassas, VA), were used. HLA-DR1 (DR B1*0101)-transgenic mice (Merck, West Grove, PA) were used at 8–10 wk of age. The chimeric HLA-DR1 molecule comprised a peptide-binding groove derived from the human DR1 sequence and a CD4-binding domain from I-A^f mice (18).

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[3] Abbreviations used in this paper: APL, altered peptide ligand; HA, hemagglutinin; CL-1, clone 1; LAT, linker for activation of T cells; F-actin, filamentous actin; ERK, extracellular signal-regulated kinase; TRITC, tetramethylrhodamine isothiocyanate; MAP, mitogen-activated protein; SLAP, Src-like adaptor protein.

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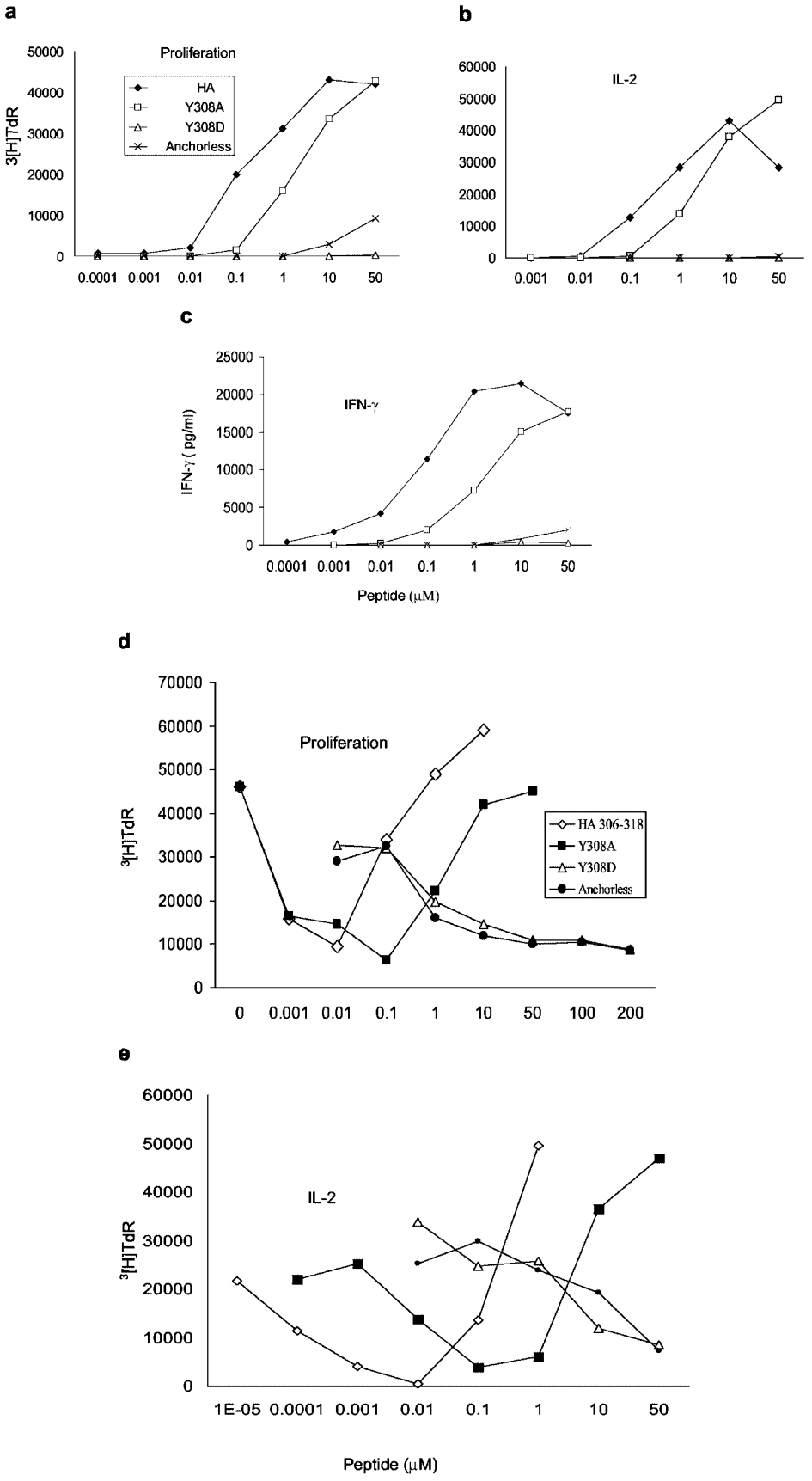

FIGURE 1. Activation and induction of anergy by short-lived variants of $HA_{306-318}$. CL-1 T cells were tested for activation by incubation with irradiated B cells and indicated concentrations of peptide analogues. Proliferation (*a*), IL-2 (*b*), and IFN-γ (*c*) production was measured. To test induction of anergy by the short-lived peptides, CL-1 T cells were incubated with irradiated B cells and various concentrations of $HA_{306-318}$ and its variants for 18 h. Then cells were washed and mixed with fresh irradiated B cells pulsed with $HA_{306-318}$ at 10 μM. Proliferation (*d*) and IL-2 production (*e*) was measured. Results are representative of at least 10 independent experiments.

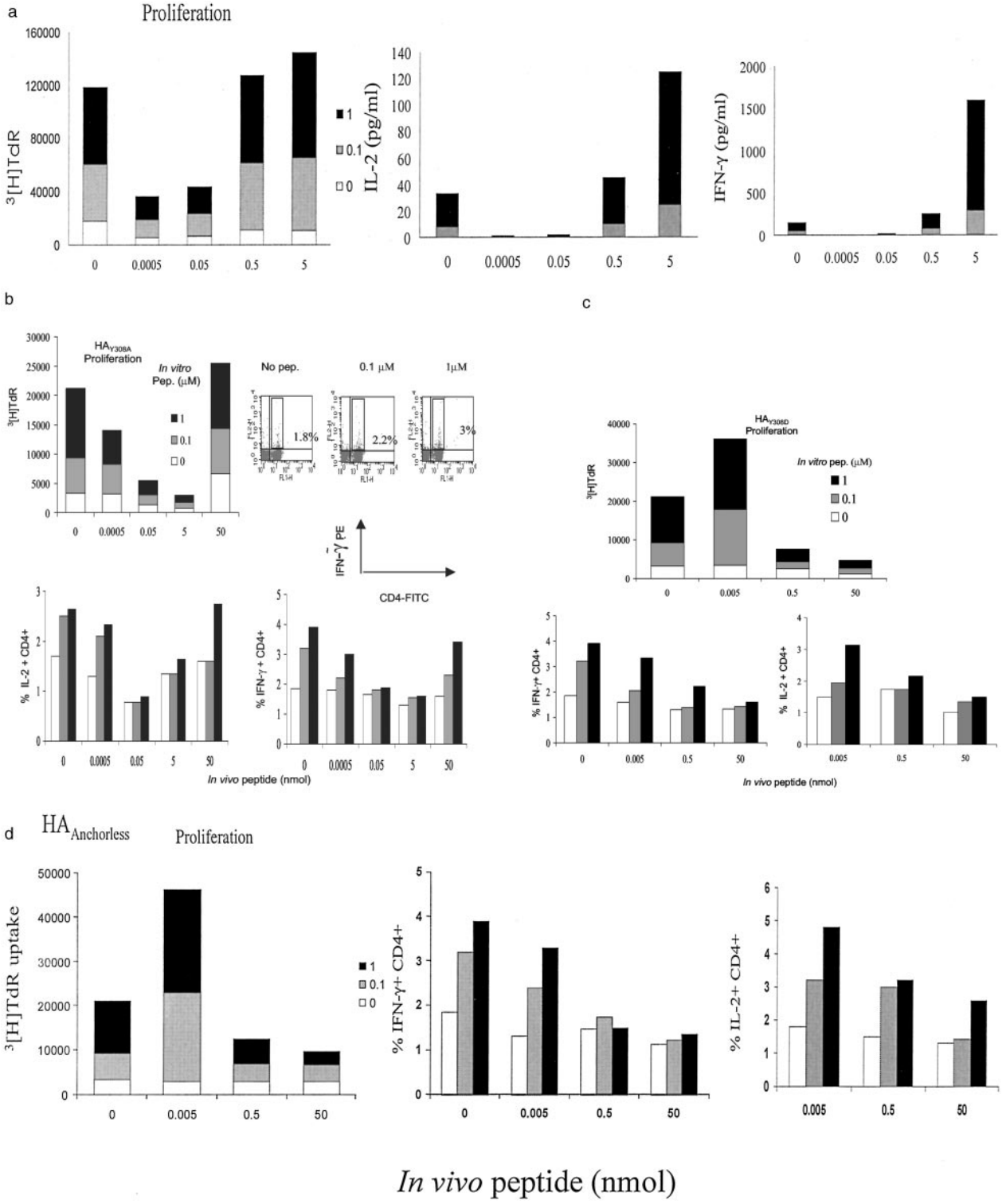

FIGURE 2. Induction of T cell anergy by short-lived $HA_{306–318}$ variants in vivo. Five weeks after immunization with $HA_{306–318}$ in CFA, mice received a second peptide injection with indicated doses of $HA_{306–318}$ (*a*), HA_{Y308A} (*b*), HA_{Y308D} (*c*), and $HA_{Anchorless}$ (*d*) in IFA. After 9 days, draining lymph nodes were collected and proliferative response to in vitro peptide restimulation was determined by [^{3}H]thymidine incorporation. IL-2 was measured by IL-2-sensitive cell line HT-2, and IFN-γ was measured by ELISA in *a* and by intracellular cytokine staining assay in *b–d*. One of three representative experiments is shown.

Peptides

The peptides, influenza virus HA, $HA_{306–318}$ (PKYVKQNTLKLAT), HA_{Y308A} (PK***A***VKQNTLKLAT), HA_{Y308D} (PK**D**VKQNTLKLAT), and $HA_{Anchorless}$ (PK**A**VK**A**N**GA**K**A**AT), were synthesized by Peptide Express (Fort Collins, CO). Bold and/or underlining indicate substituted anchor residues. The purity of the peptides was >95% as analyzed by reverse phase HPLC.

Induction of T cell unresponsiveness in CL-1 T cells

A total of 4×10^4 CL-1 T cells with 4×10^4 irradiated EBV-transformed B cells and various concentrations of peptides were cultured for 18 h at 37°C in 5% CO_2. T cells were washed. $HA_{306–318}$-pulsed, irradiated EBV-transformed B cells (10,000 rad) were then added and T cells were assayed for proliferation and cytokine production. Alternatively, either 20 ng/ml PMA (Calbiochem, La Jolla, CA), to bypass stimulation of the TCR, or 20

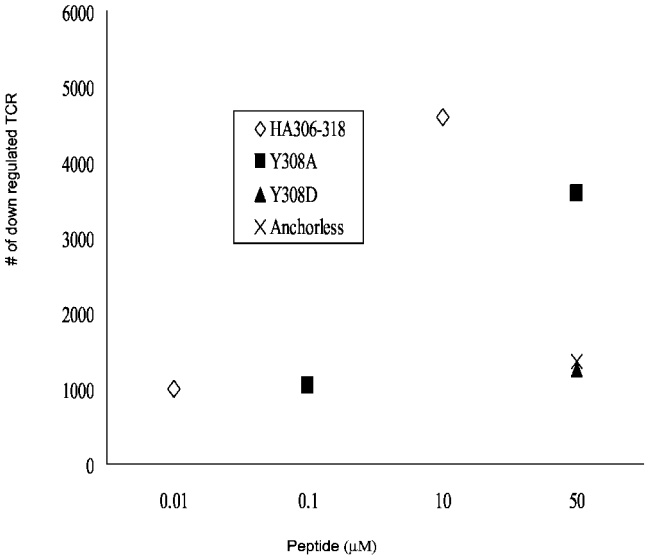

FIGURE 3. Inhibitory dose of $HA_{306-318}$ and short-lived variant peptides trigger similar levels of TCR down-regulation. A total of 10^6 CL-1 T cells was incubated with 10^6 B cells in the presence of medium alone or the indicated concentrations of peptides for 6 h. Then cells stained with TCR-CD3 complexes were enumerated by direct staining with FITC-conjugated anti-human CD3, with known numbers of FITC per molecule; fluorescence signals were assessed with a FACScan. Results are representative of five independent experiments.

IU/ml rIL-2, during or after induction of anergy, was added to the cultures. These compounds were washed out before initiation of the proliferation assay.

Induction of T cell unresponsiveness in transgenic mice

HLA-DR1-transgenic mice were immunized s.c. at the base of the tail with 10 nmol $HA_{306-318}$ peptide emulsified at a 1:1 (v/v) in CFA (Sigma-Aldrich, St. Louis, MO). Five weeks later, mice were injected with various concentrations of $HA_{306-318}$, HA_{Y308A}, HA_{Y308D}, and $HA_{Anchorless}$ in IFA s.c. Inguinal lymph nodes were removed and cells were used in proliferation assays 9 days later.

Proliferation assay

Cells (4×10^4 CL-1 T cells with 4×10^4 EBV-transformed B cells, or whole draining lymph node cells) were cultured either with no peptide or various concentrations of peptides at 37°C in 5% CO_2 for 72 h. Each well was then pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) and 18 h later, the cells were harvested (Packard Micromate Cell Harvester; Packard Instrument, Meriden, CT) and the incorporated radioactivity was measured by a Packard Matrix 96 beta counter.

Cytokine assays

T cell clones were cultured with irradiated B cells in the presence of peptides for 24–48 h. IL-2 release was measured using the IL-2-sensitive cell line HT-2 as described elsewhere (7). Cell-free culture supernatants were collected after 48 h and ELISA for mouse IFN-γ was performed according to the manufacturer's (BD PharMingen, San Diego, CA) suggested protocol. All assays were performed in triplicates.

Intracellular cytokine assay

Nine days after second peptide injection, draining lymph nodes were collected and intracellular IFN-γ production was investigated by analyzing unstimulated cells or cells stimulated with 0.1 and 1 μM $HA_{306-318}$. After incubation in 5% CO_2 at 37°C for 5 h, the protein transport inhibitor GolgiStop (BD PharMingen) was added to the cell suspension and after an additional 4 h of incubation samples were washed with staining buffer and labeled with FITC-conjugated anti-mouse CD4. Following a 30-min incubation at 4°C, cells were washed, fixed, and then permeabilized with Cytofix/Cytoperm (BD PharMingen) for 20 min at 4°C. The cells were subsequently washed twice, resuspended in 100 μl of Perm/Washing solution, and labeled with PE-conjugated anti-mouse IFN-γ for 30 min at 4°C. Cells were then washed in Perm/Wash solution and resuspended in staining buffer before analysis. Flow cytometry was performed on a FACScan (BD Biosciences, Mountain View, CA) and analyses were performed using CellQuest software.

Flow cytometry

For flow cytometry, 10^6 CL-1 T cells, 10^6 B cells, and indicated concentrations of peptides were incubated in one well of a 24-well plate for 5–18 h. The cells were then stained with FITC-conjugated anti-human CD3, PE-conjugated anti-human CD4 (BD PharMingen), PE-conjugated anti-human CD25, and anti-human CD69 (eBioscience, San Diego, CA). Quantification of TCR down-regulation was assayed as previously described (7). The samples were assayed by flow cytometry using a BD Biosciences FACS.

Immunoprecipitation and Western blotting

Briefly, 10^7 CL-1 T cells and 10^7 peptide-pulsed B cells were incubated for various periods of time (1, 5, and 10 min.) at 37°C. Activation was stopped with 2× concentrated ice-cold lysis buffer (300 mM NaCl, 20 mM NaF, 2 mM Na_3VO_4, 20 mM sodium pyrophosphate, 3 mM EDTA, 1 mM 4-(2-aminoethyl)-bezenesulfonyl fluoride · HCl, 2 μM E-64, 2 μM each aprotinin and leupeptin, 2% Nonidet P-40, and 20 mM Tris-HCl (pH 7.6)). Cells were incubated on ice for 30 min and nuclear/cytoskeletal components were removed by centrifugation at 13,000 rpm for 20 min. Cell-free lysates were subjected to immunoprecipitation with anti-phosphotyrosine mAb (PY99), polyclonal anti-ZAP-70 (Upstate Biotechnology, Lake Placid, NY), anti-linker for activation of T cells (LAT), anti-CD3ζ, anti-SLP-76, anti-Vav, and anti-Cbl (Santa Cruz Biotechnology, Santa Cruz, CA), followed by the addition of protein A/G-agarose beads and agitation by a rotary shaker for 2 h at 4°C. The beads were washed and diluted with reducing Laemmli sample buffer and then were boiled for 5 min before SDS-PAGE analysis. For detection of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (phospho-p42–44 mitogen-activated protein kinase, Cell Signaling Technology, Beverly, MA), whole cell lysates were used. Proteins and molecular mass markers (15-160 kDa; Life Technologies, Rockville, MD) were separated on 10–12% SDS-PAGE gels and then transferred to polyvinylidine difluoride membranes. The membrane was blocked by incubation in PBS containing 2% gelatin (Fisher, Pittsburgh, PA) and 0.1% Tween 20 for 1 h at room temperature and subsequently probed with anti-phosphotyrosine mAb for 1 h. The membrane was then washed three times with PBS containing 0.1% Tween 20 and was incubated further for 1 h with goat anti-mouse IgG conjugated to HRP and then washed again. Proteins were visualized by the ECL detection system (ECL plus; Amersham) and exposed to film (Kodak, Rochester, NY).

Determination of cellular filamentous actin (F-actin)

Five $\times 10^5$ B cells were pulsed with various concentrations of peptides for 2 h and than washed and mixed with 5×10^5 CL-1 T cells at different time points at 37°C with 5% CO_2. Cells were washed and stained for surface CD4 and than fixed, permeabilized, and stained with 50 ng/ml tetratmethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma-Aldrich) and F-actin content was analyzed by flow cytometry. Fifty thousand events were measured.

Results

Peptides that contain substitutions in anchor residues exhibit poor binding to DR1

Stable binding of $HA_{306-318}$ to DR1 requires the presence of an aromatic side chain (tyrosine or phenylalanine) at position 308. To study the significance of the kinetic intermediates (9, 11), we made peptide variants that had radical substitutions at this position (alanine or aspartic acid). The altered peptides, named HA_{Y308A} and HA_{Y308D}, respectively, bind poorly to DR1 and display the characteristics of kinetic intermediate complexes and do not proceed to the stable terminal complexes (14, 19–21). At 37°C these variant peptides dissociate with the half-life of 30 min or less in contrast to $HA_{306-318}$ that dissociates with a half-life of 6 days. We also designed a peptide, $HA_{Anchorless}$, that has all DR1 anchor residues substituted by alanine (positions P1, 4, 7, and 9) or by glycine (position P-6) and dissociate with a half-life of 1.5 h (19).

Activation of CL-1 T cell with substituted peptides

We tested the ability of short-lived variants of $HA_{306-318}$ at concentrations up to 50 μM to induce proliferation and IL-2 and IFN-γ production in CL-1 T cells. HA_{Y308A} induced activation of T cells, although 10-fold more HA_{Y308A} than $HA_{306-318}$ was required to achieve similar levels of activation. Only low levels of activation were induced by the $HA_{Anchorless}$ even at high concentrations, and no detectable activation was observed with HA_{Y308D} (Fig. 1, *a–c*).

Induction of T cell anergy with peptides that have a low affinity for DR1

To investigate whether short-lived variants of $HA_{306-318}$ are able to induce T cell anergy, CL-1 T cells were incubated with irradiated EBV-B cells (B cells) in the presence of various doses of the short-lived peptides for 18 h. A second set of irradiated EBV B cells, which were pulsed with 10 μM $HA_{306-318}$ for 2 h and then washed to remove unbound peptide, was added to the culture. Proliferative response and IL-2 production were assayed (Fig. 1, *d* and *e*). Short-lived $HA_{306-318}$ variants induced unresponsiveness in CL-1 T cells as determined by low levels of proliferation and lack of IL-2 production. The degree of unresponsiveness was comparable to anergy induced by low doses of $HA_{306-318}$, although higher concentrations of variant peptides were necessary to induce similar effects (concentration range, 0.1–200 μM). Importantly, short-lived peptides, HA_{Y308D} and $HA_{anchorless}$, did not induce any activation even at the highest doses of 100–200 μM, emphasizing their potential as effective tolerogens.

Effects of short-lived HA peptides in vivo

We have previously shown that presentation of low densities of high-affinity long-lived agonist peptide-MHC complexes induced anergy in peripheral memory $CD4^+$ T (8). To determine whether the short-lived $HA_{306-318}$ variants were able to induce anergy in vivo, memory T cells were induced by immunization with 10 nmol $HA_{306-318}$ in CFA. Five weeks later, memory cells were tolerized by administration of low doses of $HA_{306-318}$ and multiple doses of HA_{Y308A}, $HA_{Y308D,}$ and $HA_{Anchorless}$ peptides. Cells from the draining nodes were removed and tested in proliferation assays 9 days later (Fig. 2). As shown, low peptide doses (0.0005–0.05 nmol) induced unresponsiveness to $HA_{306-318}$ challenge in vitro. A 100-fold higher dose of HA_{Y308A} induced a similar level of unresponsiveness in vivo, consistent with the concentrations necessary for the induction of anergy in CL-1 cells. Administration of HA_{Y308D} and $HA_{Anchorless}$ peptides in IFA lead to unresponsiveness to $HA_{306-318}$ peptide at all doses examined (0.0005–50 nmol).

TCR is engaged by short-lived peptide-MHC complexes

TCR down-regulation upon Ag recognition has been established as a correlate of T cell activation (22). To test for possible engagement of TCR with short-lived peptides, CL-1 T cells were incubated with B cells with the indicated concentrations of peptides for 6 h. Cells were stained for CD3 and CD4. The number of TCR-CD3 complexes down-regulated by interaction with the short-lived peptide-DR1 complexes was determined by direct staining with FITC-conjugated anti-CD3, with known numbers of FITC per molecule followed by FACS analysis. Interestingly, we found that inhibitory doses of both the short-lived peptides and the $HA_{306-318}$ resulted in down-regulation of similar numbers ($\subseteq$1000) of TCR molecules (Fig. 3). In addition, anergy-inducing concentrations of short-lived peptides did not block CD3 down-regulation mediated by subsequent activating doses of $HA_{306-318}$ (1–10 μM; data not shown). These results indicate that the anergic state is not the result of impaired TCR/CD3 down-modulation.

As shown in Fig. 4, short-lived peptides induced similar changes in surface expression of two early indicators of T cell activation,

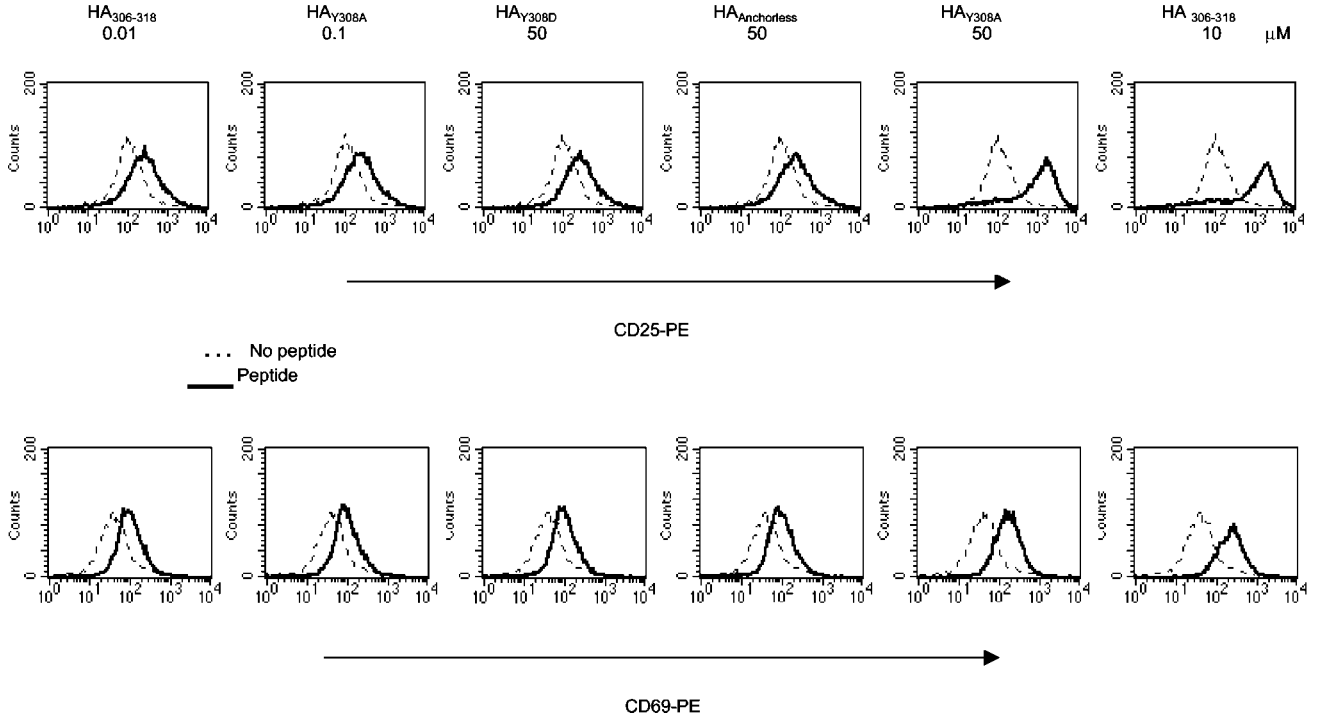

FIGURE 4. Short-lived variants of $HA_{306-318}$ peptide induce expression of activation parameters. CL-1 T cells were incubated with B cells in the absence of peptide or presence of the indicated concentrations of peptides for 5–18 h. Cells were stained with FITC-conjugated anti-human CD4 followed by PE-conjugated anti-human CD25 and anti-human CD69. Results are representative of three independent experiments.

CD25 (IL-2R) and CD69, compared with that induced by tolerogenic dose of $HA_{306-318}$. In addition, re-expression of CD25 and CD69 in response to subsequent stimulation with 10 μM $HA_{306-318}$ was not impaired (data not shown), consistent with reversal of anergy by IL-2 (either during or after the induction phase) (Fig. 5*a*). Baseline expression levels were determined by incubating T and B cells without peptide during the initial incubation phase and/or the secondary activation phase. Maximal down-regulation of CD3 and up-regulation of CD25 and CD69 was determined by incubating T and B cells without peptide during the initial phase, followed by stimulation with 10 μM HA during the secondary incubation.

IL-2 and PMA prevents/reverses the induction of anergy by short-lived peptides

We investigated the effects of IL-2 and PMA on the induction of anergy by short-lived peptides. The addition of 20 IU/ml rIL-2, or 20 ng/ml PMA, which bypasses early signaling defects, to the anergized cells restored responsiveness, suggesting that lack of IL-2 production is responsible for anergy (Fig. 5). Addition of PMA or IL-2 during the induction phase prevented induction of anergy (data not shown).

Effects of short-lived peptides on TCR-induced tyrosine phosphorylation

Ligation of the TCR leads to mobilization of the tyrosine kinases and a cascade of phosphorylation events (23) and to the expression of gene products crucial for T cell activation. To investigate the tyrosine phosphorylation in T cells stimulated with inhibitory doses of $HA_{306-318}$ and short-lived peptide variants, T cells were incubated with peptide-pulsed B cells for 1, 5, or 10 min. Cells were then lysed and subjected to immunoprecipitation with specific Abs, followed by immunoblotting with anti-phosphotyrosine. Stimulatory doses of peptides (10 μM $HA_{306-318}$ and 50 μM HA_{Y308A}) led to complete phosphorylation of the multiple immunoreceptor tyrosine-based activation motifs in the TCR-associated ζ-chains and of Syk family kinase ZAP-70, LAT, and cytosolic adaptor molecule SLP-76. The phosphorylation pattern was consistently detected up to 10 min after T cell stimulation. In contrast, stimulation of T cells with inhibitory doses of either $HA_{306-318}$ or short-lived peptides caused only a partial phosphorylation of CD3ζ and failed to induce tyrosine phosphorylation of ZAP-70 and downstream molecules, LAT and SLP-76 (Fig. 6, *a–d*). These observations held true at all three time points tested. We then examined tyrosine phosphorylation of ERK1/2 mitogen-activated protein (MAP) kinases (pp44 and pp42, respectively) and found that anergy-inducing doses of peptides led to a significant increase in phosphorylation of ERK1/2, although not at the same level of intensity to that induced by activating doses of peptides (Fig. 6*e*). ERK1/2 can be activated either by Lck/ZAP-70 or phosphorylation of Fyn (24, 25). We observed that tolerizing treatment of T cells led to full tyrosine phosphorylation of Fyn (Fig. 6*f*), suggesting that the observed pERK1/2 might be initiated by activation of Fyn.

Cbl has been shown to play a role in T cell anergy and its activation is dependent upon Fyn. Since anergic cells showed full phosphorylation of Fyn but undetectable pZAP-70, one might expect that lack of observed pZAP-70 could be due to interaction of Cbl with the negative regulatory tyrosine of ZAP-70 (26, 27). We tested whether induction of anergy could affect Cbl phosphorylation at different time points of up to 20 min. Immunoprecipitation

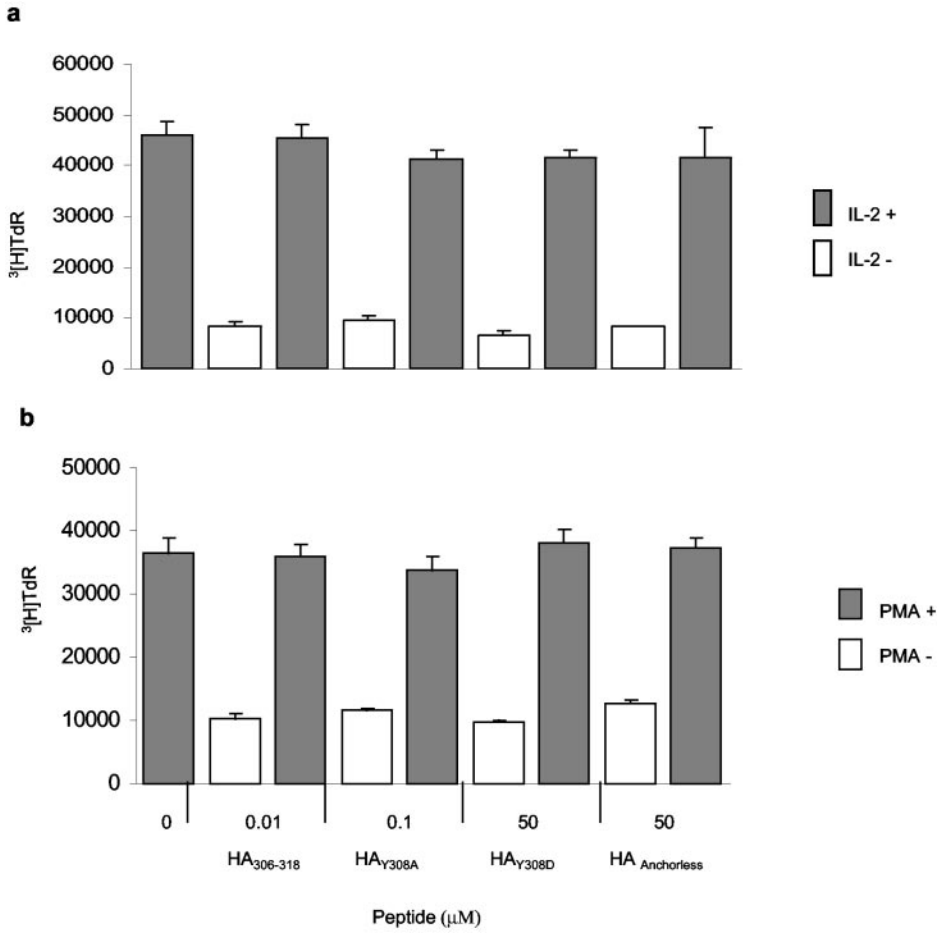

FIGURE 5. Induction of anergy by short-lived peptides is reversed/prevented in the presence of IL-2 and PMA. CL-1 cells were incubated with irradiated B cells pulsed with inhibitory doses of $HA_{306-318}$ or short-lived peptides for 18 h. Subsequently cells were washed and restimulated with $HA_{306-318}$ (10 μM)-pulsed B cells in the absence (□) or presence (▨) of either (*a*) IL-2 (20 IU/ml) or (*b*) PMA (20 ng/ml). Proliferation was assessed by [^{3}H]thymidine incorporation. Results are representative of three independent experiments.

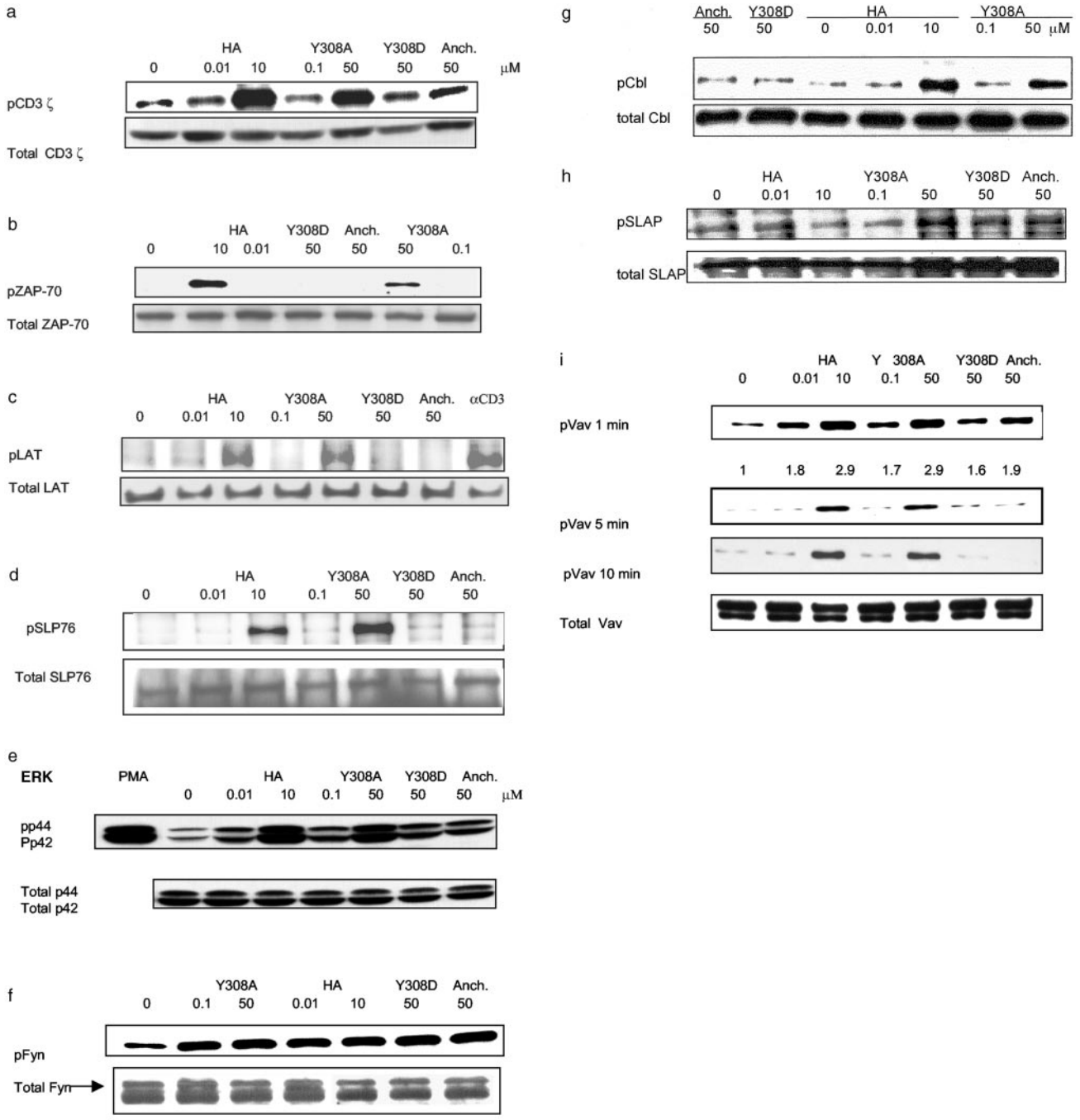

FIGURE 6. Tyrosine phosphorylation pattern of TCR-induced signaling molecules. Briefly, 10^7 CL-1 T cells were stimulated with B cells pulsed with $HA_{306-318}$ or short-lived peptides for various periods of time (1–10 min). Lysates were immunoprecipitated with anti-CD3ζ (*a*), anti-ZAP-70 (*b*), anti-LAT (*c*), anti-SLP-76 (*d*), anti-Fyn (*f*), anti-Cbl (*g*), anti-SLAP (*h*), and anti-Vav (*i*). For phosphorylation of ERK1/2 (44/42), whole cell lysates (*e*) were used. Proteins from immunoprecipitates, or whole cell lysates were resolved by reducing SDS-PAGE and transferred to membranes for immunoblotting with anti-phosphotyrosine. Immunoreactivity was detected by ECL. a–*d*, Five-minute stimulation and *e–h*, 10-min stimulation. Results are representative of two to five independent experiments.

with anti-Cbl demonstrated an increase in the tyrosine phosphorylation of Cbl in cells that were stimulated by 10 μM $HA_{306-318}$ or 50 μM HA_{Y308A} but not in those treated with inhibitory doses of peptides (Fig. 6*g*). Also, we tested whether another negative regulator of TCR signaling, the so-called Src-like adaptor protein (SLAP), which associates with CD3ζ, ZAP-70, and SLP-76 in T cells and blocks NF-AT function (28, 29) would be involved in induction of anergy by short-lived peptides. Fig. 6*h* shows no significant differences in tyrosine phosphorylation of SLAP between groups. Our data did not reveal involvement of these two negative regulatory molecules in induction of anergy by these treatments.

Vav phosphorylation is controlled by both the Ag receptor and costimulatory molecules. A preferential role of Fyn in the phosphorylation of Vav after CD28 ligation is well known (30, 31). It has been shown that Vav promotes activation of ERK, up-regulates CD69 (32, 33), and is associated with induction of anergy by APL (30). Thus, we next examined the tyrosine phosphorylation of Vav in response to tolerogenic treatment of cells. Fig. 6*i* shows that

Vav phosphorylation persisted up to 10 min in cells treated with stimulatory doses of peptides. Interestingly, tolerogenic treatments of T cells led to detection of phosphorylation of Vav only up to 1 min poststimulation. Reprobing of the stripped membranes with Abs specific for Vav verified that similar amounts of Vav protein were immunoprecipitated and loaded in all wells (Fig. 6).

Effects of short-lived peptides on actin polymerization

Specific interaction of T cell and APC results in formation of spatially segregated supramolecular activation clusters or immunological synapses at the cellular interface (34, 35) that are enriched in F-actin (36). A critical signaling molecule involved in the formation of the membrane synapse is Vav (30, 31, 37). To address whether a transient phosphorylation of Vav observed in T cells treated with inhibitory doses of peptides affects polymerization of cellular actin, T cells were mixed with peptide-pulsed B cells for 1, 3, 5, 10, and 20 min, and an intracellular polymeric form of actin (F-actin) was detected by staining with phalloidin-TRITC and analyzed by flow cytometry. We observed that T cells stimulated with tolerogenic doses of peptides did not promote F-actin polymerization even up to 20 min poststimulation (Fig. 7). In contrast, immunogenic doses of peptides led to a significant increase in F-actin content as early as 1 min poststimulation and remained at that high level up to the latest time point tested.

Discussion

Induction of tolerance in self-reactive T cells is unquestionably a highly desirable goal. Altered peptide ligands have been used either in combination with agonist peptide (1) or in preculture (2) to induce anergy in clonal T cells and for understanding the mechanisms of T cell activation. Most of these studies have focused on APLs that carry mutations at their TCR contact residues creating different topologies of peptide-MHC for interaction with specific TCR. As such, induction of anergy by APLs is generally explained by the kinetic proofreading theory, which proposes that rapid dissociation of TCR from a peptide-MHC ligand (4, 5) leads to generation of incomplete phosphorylation of the cascade of signaling molecules downstream from the TCR. However, the kinetic proofreading theory does not consistently explain the effects seen by all APLs studied (38–41) and thus the molecular mechanism of APL function has remained a mystery.

We have previously demonstrated that presentation of low densities of agonist peptide-MHC complexes induces T cell anergy. Fewer than 10 complexes of peptide-MHC per APC down-regulated 205 1000 TCR/CD3 and induced anergy in T cell clones (7) and in memory $CD4^+$ T cells in vivo (8) in several antigenic systems. We explained those finding by postulating that an overall avidity of peptide-MHC:TCR, rather than the dissociation rate of the ternary complex, is a determining factor for induction of anergy or response. To test this theory, we considered designing peptides that form short-lived complexes with MHC and might mimic intermediates in the formation of stable peptide-MHC complexes (9, 42, 43). An agonist peptide such as $HA_{306-318}$ can be converted to a peptide that can only form short-lived complexes with HLA-DR1 by substituting tyrosine (Y) 308, the main anchor residue for binding to HLA-DR1 (44), with a small or polar amino acid (19, 45). Because of rapid dissociation, complexes of substituted peptides HLA-DR1 would be displayed at low densities on the APCs. Additionally, because of a poor fit in the pocket 1, the main chain of the substituted peptides would be expected to be

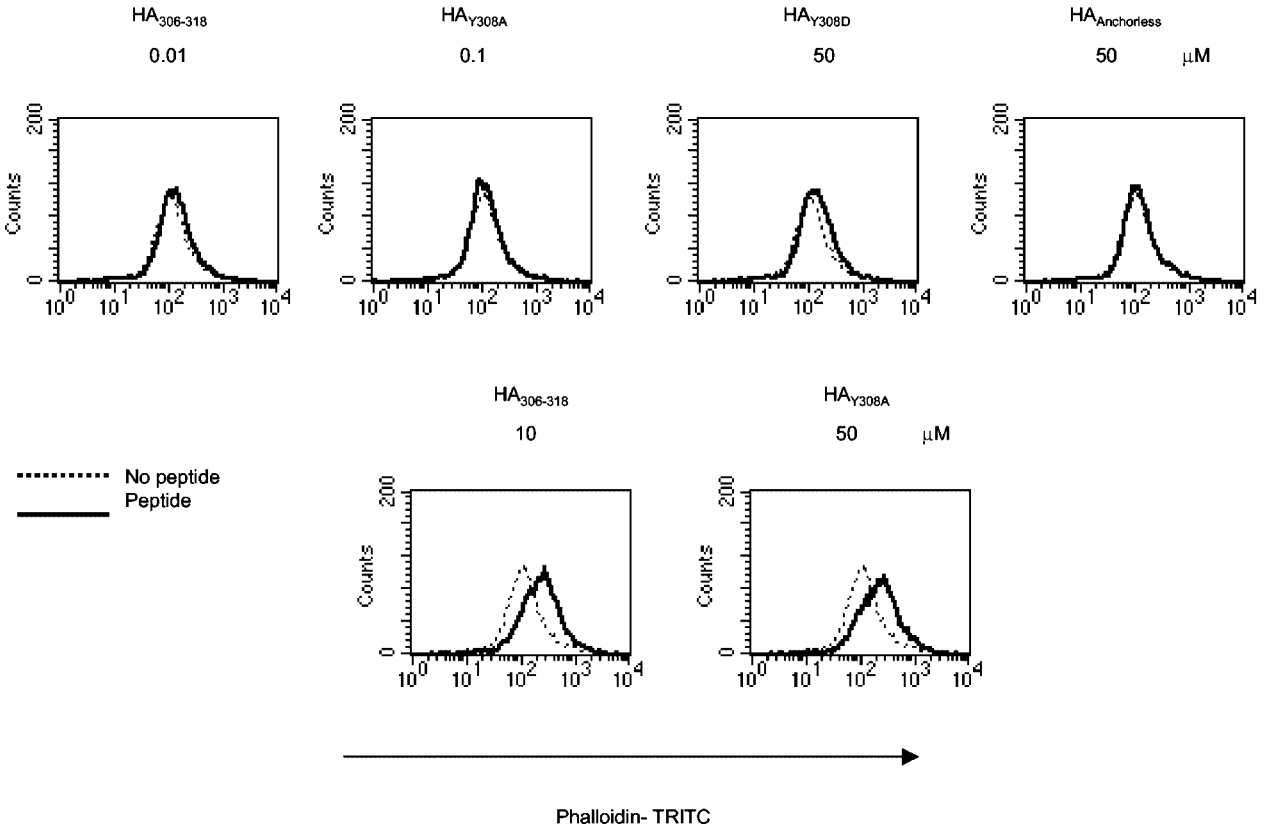

FIGURE 7. TCR ligation by inhibitory doses of peptides does not promote polymerization of cellular actin. CL-1 T cells were incubated with peptide-pulsed B cells for various lengths of time (1–20 min). Cells were then stained with FITC-conjugated anti-human CD4, followed by TRITC-phalloidin, and intracellular F-actin content was analyzed by FACS. Results are representative of four independent experiments.

more flexible than the wild-type peptide, thus reducing the overall density of the complexes that are suitably displayed (perfectly aligned) for TCR engagement (16). Consequently, short-lived complexes might behave similarly to the agonist ligands at low densities and send tolerogenic signals to the T cells. Thus, we designed short-lived peptides, such as HA_{Y308A}, HA_{Y308D}, and $HA_{Anchorless}$, and used them for the induction of anergy.

We compared short-lived peptides to agonist peptides in induction of early and late activation markers, i.e., TCR down-regulation and CD25 and CD69 up-regulation. Surprisingly, we observed that both categories of peptides induced similar activation markers at doses that induced anergy and/or activation. Among the short-lived peptides, HA_{Y308A} triggered both activation and anergy but at 10- to 100-fold higher doses than $HA_{306-318}$, whereas HA_{Y308D} and $HA_{Anchorless}$ induced only anergy. Consistent with the above prediction, HA_{Y308D} and $HA_{Anchorless}$ were more efficient in induction of anergy than HA_{Y308A} and $HA_{306-318}$ perhaps attributable to a higher flexibility due to less conserved, and/or extensive alterations at the peptide anchor residues. The major findings from these experiments were that: 1) short-lived complexes can induce anergy in T cell clones and in multiple specific clonal CD4 memory T cells in vivo, 2) anergy-inducing doses of agonist peptide and the short-lived variants stimulated similar patterns of activation markers, and 3) some short-lived peptides, despite having effective tolerizing effects, did not show any immunogenic activities even at the highest doses tested (Fig. 1*d*), emphasizing their advantage over the agonist peptide or traditional APL (46, 47) in the induction of anergy.

A parallel comparison of phosphorylation patterns of TCR signaling components indicated similar degrees of phosphorylation initiated by inhibitory doses of both categories of peptides. T cells pretreated with inhibitory doses of short-lived peptides or low doses of $HA_{306-318}$ exhibited partial phosphorylation of CD3ζ and below detection levels of pZAP-70, pLAT, and pSLP-76. However, Fyn was fully phosphorylated (Fig. 6), consistent with the phosphorylation pathway seen in anergy induced by traditional APLs (30, 48, 49)

Anergy in this system is defined by lack of IL-2 synthesis. IL-2 gene transcription is initiated by formation of an active (c-Fos and c-Jun) AP-1:NF-AT and NF-κB complexes, each an end product of different TCR/CD3-linked signaling modules. A low level of signaling transmitted by the engagement of 1000 TCR/CD3 might be sufficient to activate some but not all of the components of this transcription complex and consequently may lead to a failure in IL-2 transcription. Recent data are consistent with this explanation by showing that a constitutively active NF-AT that fails to interact with AP-1 induces expression of several anergy-associated genes in T cells (50).

Vav, a known regulator of rearrangement of the actin cytoskeleton and capping of TCR, is required for formation of the immunological synapse and for T cell activation (30, 31, 37). Vav-deficient T cells were shown to be defective in TCR-induced actin polymerization (46, 51). We observed a transient phosphorylation of Vav in T cells treated with inhibitory doses of peptides, but accompanied with no detectable actin polymerization. One might envision that anergy might occur because of formation of an immunological synapse that fails to mature (47).

Our data demonstrate that short-lived peptide-MHC complexes and low densities of long-lived agonist peptides both induce T cell anergy through engagement of fewer T cell receptors. Thus, a common trigger for the induction of anergy could be engagement of a limited number of T cell receptors by a variety of ligands. It is tempting to propose the traditional antagonist peptide ligands, having a high affinity for class II and presence in high excess relative to the agonist ligand, might simply function by occupying the majority of the available MHC molecules on the APC and reducing the number of active agonist peptide-MHC complexes to the levels just sufficient for the induction of anergy.

Use of short-lived peptides for induction of anergy is advantageous over the traditional APL: 1) because of discrimination at the level of MHC rather than at the level of TCR and the availability of many crystal structures for peptide-MHC complexes, rational design of short-lived peptide-MHC complexes is more readily attainable and 2) unlike APL that is strictly specific for a single clonal TCR, short-lived peptides can anergize several T cell clones specific for a given peptide-MHC complex in vivo. Multiple T cell clones specific for the same pair of peptide-MHC could be tolerized by this treatment as shown in HLA-DR1-transgenic mice (Fig. 2). Although APLs tolerize single T cell clones, their clinical relevance is limited when used for the treatment of pathological self-reactivity in vivo. Indeed, recent clinical trials with APLs have raised important considerations for their use for immunotherapy (52, 53). Overall, our model for the induction of anergy is an attractive approach for immunoregulation in autoimmune diseases and transplantation, and further studies should be performed in clinical situations for the purpose of immunotherapy.

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