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Immunology

Copresentation of natural HIV-1 agonist and antagonist ligands fails to induce the T cell receptor signaling cascade

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ABSTRACT **It is not known how human immunodeficiency virus type 1 (HIV-1)-derived antagonist peptides interfere with intracellular activation of cytotoxic T lymphocytes (CTL). We identified Gag epitope variants in HIV-1-infected patients that act as antagonists of CTL responses to unmutated epitopes. We then investigated the effect that presentation of each variant has on the early events of T cell receptor (TCR) signal transduction. We found that altered peptide ligands (APL) failed to induce phosphorylation of pp36, a crucial adaptor protein involved in TCR signal transduction. We further investigated the effect that simultaneous presentation of APL and native antigen at low, physiological, peptide concentrations (1 nM) has on TCR signal transduction, and we found that the presence of APL can completely inhibit induction of the protein tyrosine phosphorylation events of the TCR signal transduction cascade.**

Recognition of major histocompatibility complex (MHC)/peptide complexes by the T cell receptor (TCR) is highly specific, relying on interactions between the MHC molecule and the MHC-bound peptide with the variable regions of the TCR. Positive engagement of the TCR with an MHC/peptide complex activates the TCR-associated signal transduction cascade (1, 2). Propagation of this signal through the cell ultimately results in the activation of the various T cell effector functions. Small changes in the structure of agonist peptide can alter or diminish responses of the T cell (3). Responses to altered peptide ligands (APL) include total abrogation of functions normally elicited by agonist peptide, induction of only a subset of effector functions (4, 5), or induction of anergy in the T cell (6, 7). These effects may have important implications for understanding the function of the immune system *in vivo*. Studies in human leukocyte antigen (HLA) class II-restricted systems have identified APL that induce anergy in alloreactive CD4 helper lymphocytes, and thus may be of use in the treatment of autoimmune diseases (8, 9). In HLA class I-restricted systems it has been shown that APL can antagonize cytotoxic T lymphocyte (CTL) responses to native antigens. Naturally occurring variant peptides can antagonize responses to hepatitis B virus (10) and to the human immunodeficiency virus (HIV) (11–13). By doing so, mutant viruses harboring antagonist APL may aid the survival of wild-type virus, which otherwise would be recognized and destroyed by CTL. Thus antagonism may play an important part in the persistence of viral and perhaps other infections. Natural viral APL antagonists can exert effects at molar ratios of 0.01:1 to 0.1:1 (10, 11). Such antagonism is more potent than that achieved in other systems. It has been argued that this may be a result of positive *in vivo* selection (14).

Recent reports of MHC class I- and II-restricted antagonism have shown that TCR engagement with MHC/APL results in early intracellular signaling events that are distinct from those observed on MHC/agonist engagement. Engagement of the TCR with the MHC/agonist triggers phosphorylation of the 10 immunoreceptor tyrosine-based activation motifs (ITAMs) found in the TCR-associated CD3 and ζ_2 chains. This phosphorylation is mediated by p56Lck, a member of the Src family of protein tyrosine kinases (PTKs) associated with the cytoplasmic domain of CD4/8. Phosphorylated ITAMs recruit ZAP 70, a member of the Syk family of PTKs. ZAP 70 is then activated by Lck-mediated phosphorylation. Activated Lck and ZAP 70 interact with, and modulate the activity of, a wide variety of cytoplasmic proteins involved in intracellular signaling pathways (1, 2). Engagement of the TCR with the MHC/APL, however, leads to altered levels of ζ_2-chain phosphorylation. Subsequent recruitment by the ζ_2 chain of ZAP 70 is not followed by its phosphorylation-mediated activation (15–17). Thus it is suggested that an incomplete intracellular signal is generated, leading to an altered phenotype.

Previous reports describing the effects of APL on the early events during TCR signal transduction have concentrated on investigating the effects of agonist peptide and APL separately. Whereas antagonism of HLA class II-restricted T cell responses such as cell proliferation can be achieved by presentation of APL alone (6, 7), antagonism of CTL-mediated lysis is a phenomenon involving simultaneous presentation of both agonist peptide and APL to T lymphocytes (10, 11, 18–20). Here we address HLA class I-restricted antagonism of cell-mediated lysis. CTL lines from peripheral blood lymphocytes of HIV-1-infected individuals against HIV-1 HLA B8- and HLA A2-restricted Gag epitopes were cultured. We used these lines to investigate the effect on the early events of TCR signal transduction of copresentation of native epitope and naturally occurring antagonist at physiological concentrations.

METHODS

Peptides, Antibodies, Glutathione *S*-Transferase (GST) Fusion Proteins, and Reagents. Peptides were synthesized with standard Fmoc chemistry and purified by reverse-phase HPLC. Anti-phosphotyrosine mAb clone 4G10 and anti-Lnk mAb were purchased from TCS Biologicals (Botolph Claydon,

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Abbreviations: APL, altered peptide ligand(s); CTL, cytotoxic T lymphocyte(s); HLA, human leukocyte antigen; HIV-1, human immunodeficiency virus type 1; MHC, major histocompatibility complex; TCR, T cell receptor; GST, glutathione *S*-transferase; APC, antigen-presenting cells.

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U.K.). Horseradish peroxidase-linked anti-mouse Ig antibody was purchased from Amersham. The anti-p38 mitogen-activated protein kinase (MAPK) antibody was purchased from New England Biolabs. The GST-Grb2 fusion protein has been previously described (21). All reagents were purchased from Sigma–Aldrich unless indicated otherwise.

Cell Lines. The HLA B8 Gag p17-3-specific CTL line was cultured from the peripheral blood mononuclear cells of HIV-1-infected patients as previously described (22). Generation of the HLA A2 Gag p17-8 CTL line was similar, except that after initial exposure of bulk culture to A2 Gag peptide (SLYNTVATL) cells were fed every third week with a 1:1 mixture of irradiated [3,000 rads (1 rad = 0.01 Gy)], phytohemagglutinin (2 μg/ml)-treated peripheral blood mononuclear cells and were not presented with peptide antigen. B cell lines were immortalized and cultured as previously described (23).

CTL Activation. B cells were used as antigen-presenting cells (APC). CTL and APC were washed twice in RPMI medium 1640 to remove fetal calf serum. To present antigen, HLA-matched APC were pulsed with peptide for 2 hr at 37°C. Peptide-pulsed APC were presented to CTL at a ratio of 1:10 in a final volume of 50 μl. Presentation of peptide-pulsed B cells to CTL was for 10 min at 37°C.

Immunoblotting. After activation of 10^6 CTL, cells were pelleted for 30 sec at $7,000 \times g$. Cells were lysed in 20 μl of lysis buffer [10% (vol/vol) glycerol/1% Nonidet P-40/140 mM NaCl/20 mM Tris·HCl, pH 8.0/10 mM NaF/2 mM EDTA containing1 mM sodium orthovanadate, 25 μM *p*-nitrophenyl *p′*-guanidinobenzoate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Lysate was spun in a Microfuge for 15 min at 13,000 rpm to pellet the nuclear fraction. Then 5 μl of reducing buffer [125 mM Tris·HCl, pH 6.6/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol/4% SDS/0.25% bromophenol blue] was added to the supernatant, and samples were boiled for 5 min to denature protein. Proteins were separated overnight by SDS/PAGE on a 12% acrylamide gel (Ultra Pure Protogel; National Diagnostics). Protein was transferred onto Hybond-ECL nitrocellulose (Amersham) by a semidry Western blotting system. The nitrocellulose membrane was blocked for 2 hr at 4°C with 1% blot-quality BSA (ProtoBlot BSA; Promega) in PBS-Tween (0.1% Tween-20 in PBS) and probed with anti-phosphotyrosine mAb (1 μg/ml, 0.1% BSA in PBS-Tween) overnight at 4°C. Nitrocellulose was washed four times for 10 min each with PBS-Tween and incubated with horseradish peroxidase (HRP)-linked antibody to mouse Ig (1:10,000 dilution in 2.5% dried milk/PBS-Tween) for 2 hr at 4°C. HRP activity was detected by enhanced chemiluminescence (ECL; Amersham).

Protein Precipitation with GST Fusion Protein. HLA A2 Gag p17-3 CTL (3×10^6) were activated by presentation of A2 Gag peptide (SLYNTVATL) and lysed as described. Lysate was diluted to 100 μl with lysis buffer lacking Nonidet P-40. Lysate was precleared with 20 μl of 50% glutathione-Sepharose slurry (GSH-Sepharose; Pharmacia Biotech). Precleared lysate was incubated overnight with 40 μl of 50% GSH-Sepharose slurry and 25 μg of GST-Grb2 fusion protein. Sepharose was pelleted in a Microfuge and the cleared lysate was removed. The pellet was washed five times in lysis buffer containing 0.1% Nonidet P-40 and resuspended in 40 μl of glutathione elution buffer (10 mM reduced glutathione/50 mM Tris·HCl, pH 8.0). Eluate and the cleared lysate were electrophoresed and anti-phosphotyrosine immunoblotted as described above.

RESULTS

Presentation of Naturally Occurring APL of HIV-1 Epitopes Fails to Induce the Full Protein Tyrosine Phosphorylation Cascade in CTL After TCR Ligation. Antagonism by naturally occurring APL of CTL responses to HLA B8-restricted HIV-1 Gag 17-3 (GGKKKYKL) antigen has been previously described (11–13). We have also observed APL-induced antagonism of CTL-mediated lysis to an HLA A2-restricted HIV-1 Gag p17-8 epitope (SLYNTVATL) (19). These APL included partial agonists/antagonists and a strict antagonist. The sequences of agonist peptides and APL are summarized in Table 1. Previous work by others indicates that antagonism involves early events of intracellular signaling rather than later events such as intracellular calcium release (15–17). As these proximal events in intracellular TCR signaling involve activation of protein tyrosine phosphorylation cascades, we studied the effect of APL presentation on the protein phosphotyrosine profile within HIV-1-specific CTL.

Presentation of agonist peptide-pulsed APC to CTL elicits a clear intracellular biochemical signal in the form of increased levels of protein tyrosine phosphorylation in comparison to levels in resting CTL. The increase ranges from modest in the HLA B8-restricted system (Fig. 1*a*) to manyfold in the HLA A2-restricted system (Fig. 1*b*). Presentation of unpulsed targets to CTL results in a slight increase of protein tyrosine phosphorylation in comparison to resting CTL (Fig. 1*b*). MHCs on unpulsed targets are occupied by self-derived peptides that are not recognized by the CTL lines. The activation seen is presumably due to intracellular signals generated by interactions between surface coreceptors of the target and CTL or by peptide-independent TCR–MHC interactions (24, 25). A signaling response identical to that when unpulsed targets were presented to CTL was seen when targets were pulsed with an extraneous peptide epitope, such as HLA A2-restricted influenza virus matrix protein or HTLV-1 Tax CTL epitopes (data not shown). In the HLA B8-restricted system the general protein phosphotyrosine levels in response to APL are similar to levels seen in response to the agonist peptide. The exception was a protein of 36 kDa that was phosphorylated only on presentation of agonist peptide (Fig. 1*a*). The CD3 ζ_2-chain was observed to be constitutively tyrosine phosphorylated in its 21-kDa isoform, and levels of phosphorylation did not appear to be significantly altered after presentation of either APL or agonist peptide.

In the HLA A2-restricted system, general protein tyrosine phosphorylation levels in response to APL are measurably higher than in response to unpulsed targets, but substantially

Table 1. Summary of HIV-1 epitopes and naturally occurring APL

Peptide epitope	Peptide Seq	HLA	Phenotype
HIV-1 17-3 Gag (Index)	GGKKKYKL	B8	Agonist
HIV-1 17-3 Gag (7R)	GGKKKY**R**L	B8	Agonist/Antag.
HIV-1 17-3 Gag (7Q)	GGKKKY**Q**L	B8	Agonist/Antag.
HIV-1 17-3 Gag (7A)	GGKKKY**A**L	B8	Untested
HIV-1 17-8 Gag (Index)	SLYNTVATL	A2	Agonist
HIV-1 17-8 Gag (3C)	SL**C**NTVATL	A2	Agonist/Antag.
HIV-1 17-8 Gag (3S)	SL**S**NTVATL	A2	Agonist/Antag.
HIV-1 17-8 Gag (3F, 6I, 8V)	SL**F**NT**I**A**V**L	A2	Agonist/Antag.
HIV-1 17-8 Gag (3F, 5A)	SL**F**N**A**VATL	A2	Strict antag.

CTL lines were grown from patients' peripheral blood mononuclear cells against the index peptides. Proviral sequence from patients mounting an HLA B8 p17-3 Gag (11) or HLA A2 p17-8 Gag (19) response revealed several codon-changing mutations within these epitopes. The effect of naturally ocurring APL on CTL-mediated lysis in response to index peptide was evaluated in chromium release killing assays. Partial agonists/antagonists (Agonist/Antag.) are defined as peptides that at low concentrations ($<10^{-7}$ M; ref. 19) inhibit CTL-mediated lysis in response to agonist peptide, but at higher concentrations ($>10^{-6}$ M) elicit lysis against themselves. Strict antagonists are defined as peptides that inhibit CTL-mediated lysis in response to agonist peptide but do not elicit lysis against themselves at any concentration measured (18). Underlined letters indicate MHC anchor residues; letters in boldface indicate variant amino acid.

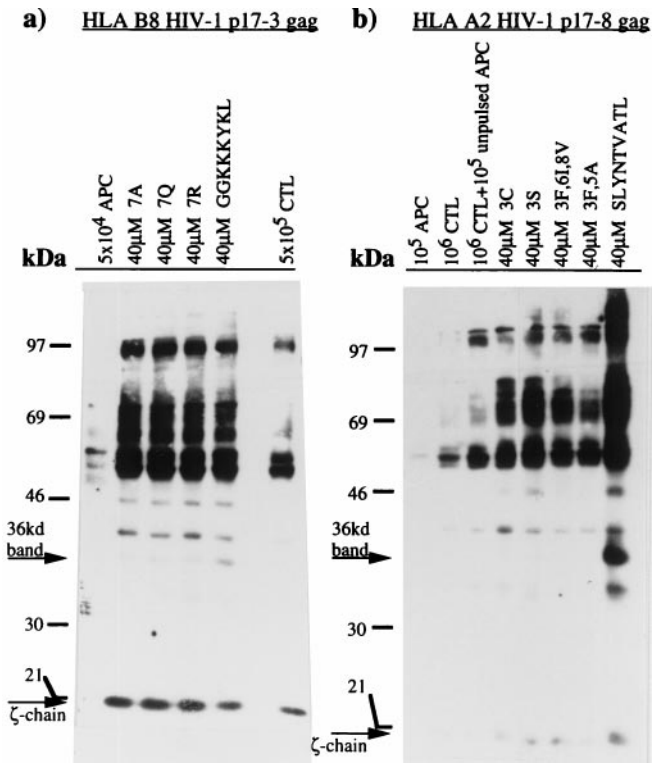

FIG. 1. Different patterns of protein tyrosine phosphorylation are observed 10 min after presentation of APLs or agonist peptide. (*a*) CTL (5×10^5) from an HLA B8-restricted line recognizing HIV-1 p17-3 Gag from patient no. 84 were presented for 10 min with 5×10^4 APC pulsed with agonistic peptide (GGKKKYKL), partial agonist/antagonist (7R or 7Q), or a natural APL of unknown phenotype (7A) at 40 μM. Cell lysates were electrophoresed and immunoblotted with anti-phosphotyrosine mAb. Resting CTL were used as a control. Protein tyrosine phosphorylation levels induced in CTL by unpulsed APC were significantly lower than those induced by peptide-pulsed APC (data not shown). (*b*) CTL (10^6) from an HLA A2-restricted line recognizing HIV-1 p17-8 Gag from patient no. 868 were presented for 10 min with 10^5 APC pulsed with agonist peptide (SLYNTVATL), strict antagonist (3F, 5A), or partial agonist/antagonist (3C or 3S or 3F, 6I, 8V) at 40 μM. CTL presented with unpulsed APC were used as a control. Cell lysates were electrophoresed and immunoblotted with anti-phosphotyrosine mAb.

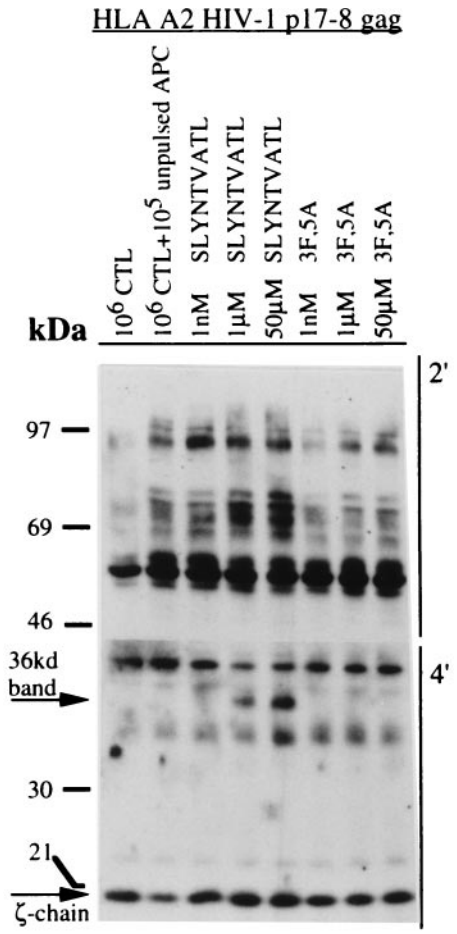

FIG. 2. Protein tyrosine phosphorylation in response to titrations of agonist peptide and antagonist APL. CTL (10^6) from an HLA A2-restricted line recognizing HIV-1 p17-8 Gag from patient no. 868 were activated by presenting with 10^5 APC pulsed with either agonist peptide (SLYNTVATL) or the strict antagonist (3F, 5A) at the concentrations indicated above each lane (1 nM, 1 μM, or 50 μM). CTL presented with unpulsed APC were used as a control. Cell lysates were electrophoresed and immunoblotted with anti-phosphotyrosine mAb. For the most detailed depiction, the figure was composed of a 2-min and a 4-min ECL exposure of the same immunoblot.

lower than the phosphorylation seen after exposure to agonist peptide (Fig. 1*b*). APL-induced protein tyrosine phosphorylation correlated inversely with the ability of each to act as a TCR antagonist (19)—i.e., weak antagonists induced a more complete profile, and greater levels, of protein tyrosine phosphorylation. Of all the APL the strict antagonist (3F, 5A) induced the weakest, barely detectable, increase in protein tyrosine phosphorylation. The 36-kDa protein was phosphorylated only on presentation of agonist peptide (Fig. 1*b*). Though the CD3 ζ_2 chain was again observed to be constitutively tyrosine phosphorylated, levels of phosphorylation increased significantly after presentation of agonist peptide to CTL. The effect of agonist peptide or antagonist APL (3F, 5A) on induction of protein tyrosine phosphorylation (Fig. 2) was titrated to nanomolar levels. We usually observed the onset of both the intracellular biochemical events and the cytolytic response (19) to agonist peptides near the lower end of this range (1–10 nM). Agonist peptide induced protein tyrosine phosphorylation in a dose-responsive manner, whereas the strict antagonist APL induced no or barely detectable protein tyrosine phosphorylation at any peptide concentration measured. Again, the 36-kDa protein was phosphorylated only in response to agonist peptide.

Copresentation of Agonist and Antagonist Ligands Fails to Induce the Full Protein Tyrosine Phosphorylation Cascade in CTL After TCR Ligation. Classically, antagonism of CTL-mediated lysis depends on the simultaneous presentation of agonist peptide and APL (18). Recent work in our laboratory on the HLA A2 HIV-1 Gag p17-8-restricted CTL line as well as similar studies by others have shown that presentation of antagonist APL alone does not diminish the subsequent cytotoxic response of CTL to native antigen (19, 20). Therefore, APL do not generate a lasting negative signal anergizing the ability of CTL to mediate lysis. The antagonism phenotype is thus dependent on the simultaneous presentation to CTL of APL and agonist peptide. Because the effects on intracellular signaling of such a combination have not been previously studied we investigated the effects of the combination of peptides on early protein tyrosine phosphorylation events in CTL. We made serial comparisons of the extent of tyrosine phosphorylation in CTL exposed to targets either presenting agonist peptide alone or copresenting agonist peptide and antagonist APL. We were able to expand the HLA A2 HIV-1 p17-8 Gag-restricted CTL line to the numbers needed for further analysis.

Targets pulsed with either 1 μM agonist peptide or, at a more physiological concentration (26), with 1 nM agonist

peptide induced a protein tyrosine phosphorylation cascade that peaked at 10 min (Fig. 3). A minimal increase in protein tyrosine phosphorylation was observed when both agonist peptide and antagonist APL were copresented (1:1). Copresentation of agonist peptide and APL never induced a profile of protein tyrosine phosphorylation comparable to that induced by agonist peptide alone. Tyrosine phosphorylation of the CD3 ζ_2 chain increased, and peaked at 10 min, after presentation of agonist peptide alone, but it remained at basal levels throughout the time course when agonist peptide and APL were copresented. Tyrosine phosphorylation of the 36-kDa protein increased marginally on copresentation of APL and agonist peptide, in keeping with the overall small increase in protein tyrosine phosphorylation observed under these conditions.

We repeated this experiment with a different HIV-1 Gag p17-8-restricted CTL line, which is not antagonized by the 3F, 5A variant (19). In this case simultaneous presentation of the 3F, 5A variant did not interfere with induction of the tyrosine phosphorylation events in response to agonist peptide (data not shown).

The 36-kDa Protein That Is Phosphorylated in Response to Agonist Peptide but Not to APL Is the Phosphoprotein Known as pp36. We surmised that the protein of 36 kDa that is tyrosine phosphorylated only in a CTL response to agonist peptide, but not to APL alone, may play a crucial role in induction of the cytotoxic response. A protein of 36 kDa (pp36) that is tyrosine phosphorylated upon TCR crosslinking and that interacts with many components of the TCR signal transduction cascade (27–30) has been reported on several occasions (2). pp36 may help coalesce parts of the signaling cascade and position them near the membrane-bound components of the TCR/CD3 complexes. A recent report has linked inhibition of natural killer (NK) cell cytotoxic responses to nonphosphorylation of pp36 (31). Nonphosphorylation of pp36 presumably disrupts the ability of pp36 to form a complex with Grb2 and phospholipase C-γ1 (PLCγ1). Tyrosine phosphorylation of PLCγ1 remains unaffected, but formation of the trimolecular complex seems necessary for PLCγ1-induced generation of inositol 3-phosphate *in vivo*. The resulting disruption of the calcium signaling pathway presumably prevents induction of the cytotoxic response. pp36 is known to bind to Grb2 (21). We used a GST-Grb2 protein (21) to precipitate the tyrosine-phosphorylated band of 36 kDa (Fig. 4), so demonstrating that the 36-kDa band was indeed the pp36 involved in the intracellular TCR signaling cascade. It has been proposed that pp36 may be the human homologue of Lnk, a protein of 36 kDa expressed in rat lymphocytes that is able to interact with components of the TCR signal transduction cascade (32). Using an anti-Lnk mAb that crossreacts with a human protein of ≈36 kDa, we have shown that pp36 is not the human homologue of Lnk (data not shown). This conclusion agrees

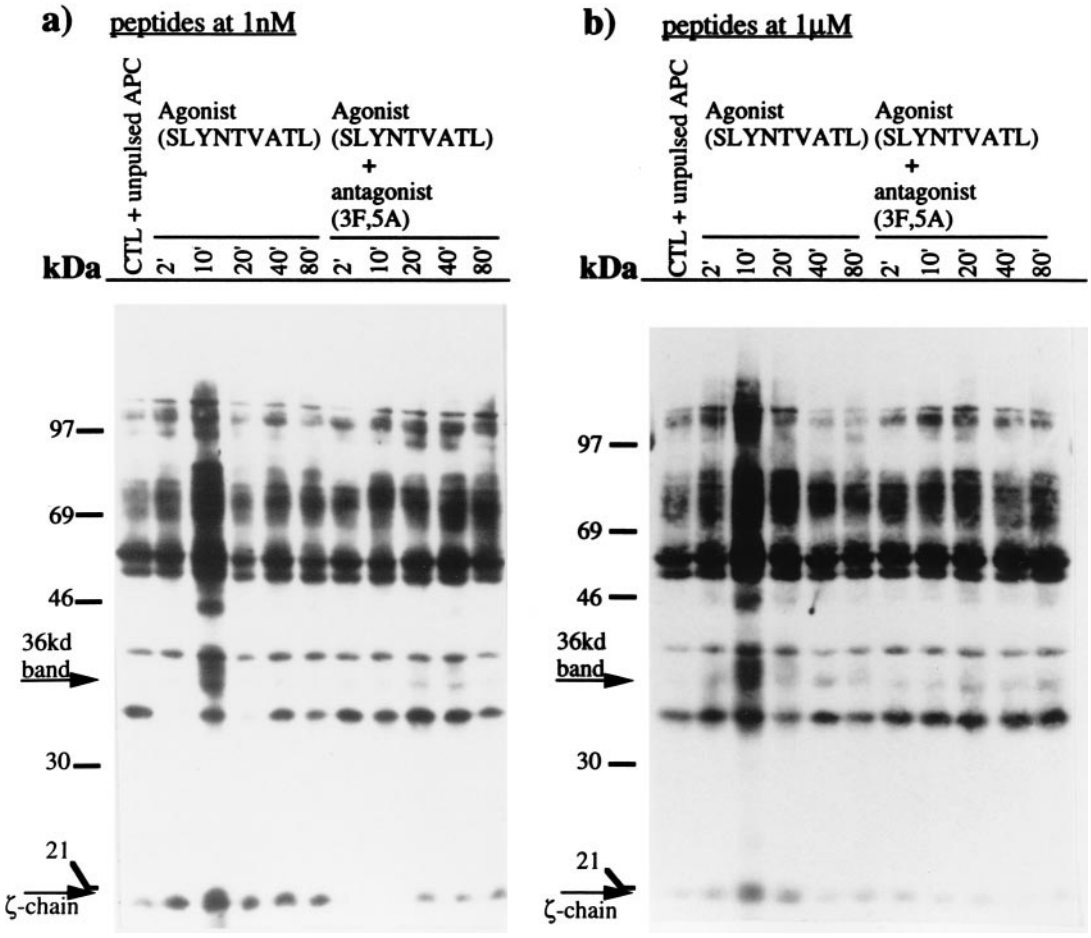

FIG. 3. Presence of antagonist APL prevents induction of complete protein tyrosine phosphorylation profile in response to agonist peptide. (*a*) CTL (10^6) from the HLA A2-restricted line recognizing HIV-1 p17-8 Gag were presented for different times (2, 10, 20, 40, or 80 min) with 10^5 APC either presenting agonist peptide alone or copresenting agonist peptide (SLYNTVATL) and antagonistic APL (3F, 5A). To present peptide, APC were pulsed with 1 nM agonist peptide for 2 hr, washed with RPMI medium 1640, and then either pulsed for a further 2 hr with 1 nM APL (for copresentation) or resuspended in the absence of peptide for 2 hr (for agonist presentation alone). Cell lysates were electrophoresed and immunoblotted with anti-phosphotyrosine mAb. The disappearance of the ζ_2 chain observed at 2 and 10 min during agonist and APL copresentation was not observed in subsequent experiments, and is most likely an artifact of Western blotting. Data are representative of at least four experiments. (*b*) As in *a*, except that peptides were used at 1 μM. Data are representative of at least four experiments.

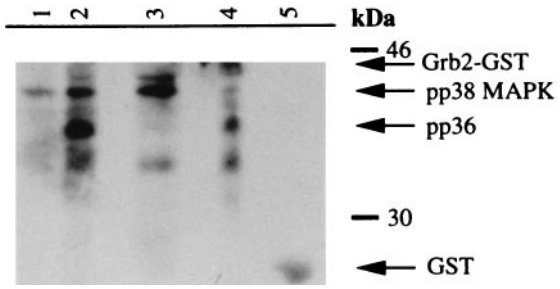

FIG. 4. Precipitation of the 36-kDa band from activated cell lysates with a GST-Grb2 fusion protein. CTL (3×10^6) from the HLA A2-restricted line recognizing HIV-1 p17-8 Gag were activated by presenting with 3×10^5 APC pulsed with 50 μM agonist peptide (SLYNTVATL). Either a GST-Grb2 fusion protein or GST protein was added to the cell lysate, and fusion protein complexes then were precipitated as described in the text. Proteins in lysate cleared by GST-Grb2 (lane 3), in the GST-Grb2 precipitate (lane 4), and in the GST precipitate (lane 5) were separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine mAb. Untreated detergent-soluble fractions from lysates of resting (lane 1) and activated (lane 2) CTL were run as controls. The band of 38 kDa that weakly bound to the GST-Grb2 fusion protein was identified as phospho-p38 mitogen-activated protein kinase (MAPK) by reprobing the blot with anti-p38 MAPK Ab (data not shown).

with recent data from Lnk knockout mice that distinguish Lnk from pp36 (33).

DISCUSSION

These experiments show that anti-HIV CTL can be antagonized by physiologically plausible concentrations of naturally occurring APL. When CTL engage these variant antigens, tyrosine phosphorylation of the key signaling molecule pp36 does not occur. We have extended these studies to include situations where a strictly antagonistic APL and the wild-type epitope are copresented to CTL at physiological concentrations. We show that the presence of the APL interferes with the induction of the tyrosine phosphorylation events during TCR signal transduction.

Models of TCR Antagonism. Three models have been put forward to explain the effects of APL on T cell effector function. In the competitive model the MHC/APL complex competes with the MHC/agonist peptide complex for the ligand binding site of the TCR in a traditional competitive fashion, so inhibiting the formation of signal-inducing MHC/agonist peptide/TCR complexes. However, because antagonism can occur at low APL-to-agonist peptide ratios (10, 19, 34) and because TCR affinity for MHC/APL is typically one order of magnitude lower than for the MHC/agonist peptide complex (35, 36), the argument for the competitive model of TCR antagonism is hard to sustain. The second model is a structural model. In it the TCR, or the TCR/CD3/ζ_2/CD4/8 multiprotein complex, has to oligomerize and/or undergo a conformational change to induce an intracellular signal. APL may prevent proper oligomerization or induce an alternative conformation (37, 38). This either leaves other elements of the signaling cascade unable to bind to the altered conformation, and thus inhibits any further propagation of the signal, or it may lead to the generation of a negative intracellular signal. The third model is the kinetic discrimination model (39, 40). In it, reduced time of occupancy of the TCR by the MHC/APL leads to a reduced, incomplete intracellular signal. In support of this model are studies which show that MHC/APL complexes tend to have lower affinities for the TCR and faster dissociation rates from the TCR than do MHC complexes with native antigen (35, 41). MHC/APL complexes with different TCR occupancy times and affinities are predicted to induce intracellular signals of different "completeness".

Signals Induced by APL. The first feature of the investigated APL is that they induce an incomplete intracellular signal in CTL in the form of an altered profile of protein tyrosine phosphorylation. Previous studies have shown that APL induce incomplete phosphorylation of the CD3 ζ_2 chain (15–17). Recent studies of HLA class II-restricted responses have shown that different APL can have differential effects progressively further downstream into the signaling cascade (42), starting at ζ_2-chain phosphorylation, then partial calcium flux, acid release, full calcium release, and finally cell proliferation. These results support the concept of a hierarchy of "completeness" of signals predicted by the kinetic discrimination model of TCR antagonism.

The APL we have described induce limited activation of the tyrosine phosphorylation events in CTL. We do not observe the production of differential ζ_2-chain phosphoforms upon presentation of APL (Figs. 1–3). Indeed, the ζ_2 chain appears to be constitutively phosphorylated (Fig. 1*a*). This conclusion is consistent with reports that the ζ_2 chain is constitutively phosphorylated and associated with ZAP 70 in T lymphocytes *in vivo* (43, 44), and cell lines may lose this trait after prolonged culturing. Instead our APL specifically fail to induce the tyrosine phosphorylation of pp36, while increasing levels of protein tyrosine phosphorylation in general. Phosphorylation of pp36 falls in between ζ_2 phosphorylation and partial calcium flux in the hierarchy of T cell activation. Thus the HIV-1 APL we have identified are capable of inducing a signal more complete than that leading to partial ζ_2-chain phosphorylation but that falls far short of generating a complete signal. This effect is compatible with the kinetic discrimination model of APL-induced signal transduction.

Agonist and Antagonist Ligand Copresentation. The second feature of the investigated APL is their ability to antagonize the cytotoxic response to simultaneously presented agonist peptide (19). We show that the antagonistic effect is mediated by inhibition of the agonist peptide-induced protein tyrosine phosphorylation events. This inhibition is virtually complete, with only barely detectable increases in phosphotyrosine levels observed on copresentation. In CTL whose cytotoxic response to agonist peptide is not subject to antagonism by the described APL (19), induction of protein tyrosine phosphorylation too is not affected by the presence of antagonist. We and others have previously shown that APL alone do not generate a lasting negative signal anergizing CTL ability to mediate lysis (19, 20). Thus the altered biochemical events we observe in CTL after presentation of APL alone do not represent an anergizing signal. Simultaneous presentation of APL and agonist peptide is therefore required for antagonism of CTL-mediated lysis. It is possible that the signal generated by presentation of APL may be a short term inhibitory one, with copresentation of agonist peptide failing to overcome the APL-induced disruption of the signaling cascade. However, it remains difficult to see how the kinetic discrimination model can account for the destructive interference by the APL-induced signal on the simultaneous propagation of the agonist peptide-induced signal. Alternatively, the APL-induced intracellular signal may be inconsequential to antagonism (45). It may be expected that APL induce an intracellular signal, however truncated, as by definition they are structurally very similar to the agonist peptide. Indeed, at high enough concentrations many APL will induce CTL-mediated lysis (18, 19). Antagonism may instead result from interference by MHC/APL with productive clustering of the MHC/agonist and TCR, thus preventing the induction of any intracellular signal. It has been shown that TCR cluster into multimers after engagement with MHC/peptide (38), with cluster formation being a prerequisite for the generation of an intracellular signal (46). The MHC/APL complex may interact with the TCR long enough to be incorporated alongside MHC/agonist into clusters, leading to an unproductive outcome.

Our results have implications for the models describing antagonism of CTL-mediated lysis. The kinetic discrimination model successfully describes the nature of the APL-induced intracellular signal, and thus may be invoked to describe forms of antagonism induced by APL alone, such as T cell anergy (6, 7). It remains to be seen whether the kinetic discrimination model can be successfully employed to model the destructive interference APL can have on agonist peptide-induced intracellular signals. Conformational models suggest that APL interfere with a structural aspect of agonist peptide-induced receptor oligomerization. Whilst this would adequately explain our observations, such models remain largely uninvestigated experimentally.

Note Added in Proof. During the processing of this manuscript a gene for a protein involved in the activation of T cells (*LAT*, linker for activation of T cells) that runs at an apparent molecular mass of 36 kDa on SDS/PAGE has been cloned. The authors identify this as the protein commonly referred to as pp36 (47).

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