



**Prevention of HIV-1 Infection and Preservation of CD4 Function by the Binding of CPFs to gp120**

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  22. The results shown in Figs. 1 to 4 are typical of those from several independent preparations. P13.1 cells, for example, were extracted on three occasions; BALB.B male cells were extracted more than ten times. In all cases, the elution profiles as well as the position of CTL-recognized material were remarkably similar.
  23. To make sure that the detection of  $\beta$ -galactosidase-derived CTL-epitopes in cell extracts is not due to fragmentation during the preparation, we suspended 1 mg of *E. coli*  $\beta$ -galactosidase (G-2513, Sigma) in 20 ml of 0.1% trifluoroacetic acid (TFA), dounced, and sonicated as described for the cell extracts. After two rounds of lyophilization, the material was resuspended in 0.1% TFA. The centrifuged supernatant was set aside, and the pellet was dried by speed-vac centrifugation, digested with pepsin (P-7012, Sigma) (1 ml, 0.1 mg/ml, 50 mM sodium acetate, pH 2.5, for 3 hours at 37°C), lyophilized, and suspended in 1 ml of 0.1% TFA. Both the soluble TFA extracts of undigested and digested  $\beta$ -galactosidase were subjected to reversed-phase HPLC exactly as described for the cell extracts. Fractions were dried and tested for recognition by CTL. As can be seen in Fig. 1, A and C, TFA-treatment of  $\beta$ -galactosidase did not lead to the production of fragments or CTL-epitopes.
  24. Eluant A, 0.1% TFA; eluant B, acetonitril containing 0.1% TFA; gradient, 0 to 60% B; flow rate, 1 ml/min; fraction size, 1 ml.
  25. CTL assays were performed according to standard methods (7). Briefly,  $^{51}\text{Cr}$ -labeled P815 tumor cells were incubated in a total volume of 150  $\mu\text{l}$  of a fraction diluted 1:3 in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) (Gibco), containing 10% fetal bovine serum, for 90 min at 37°C. Effector cells (50  $\mu\text{l}$ ) were added, and the assay was incubated for 6 hours (37°C, 5%  $\text{CO}_2$ ). Radioactivity released into the supernatant was determined in a gamma-counter. Spontaneous release of target cells ranged between 12 and 16%; the effector-to-target ratio was between 1:10 and 1:30.
  26. Marker proteins: 66 kD, bovine serum albumin; 21.5 kD, soybean trypsin inhibitor; 3.3 kD, secretin; 1.4 kD, synthetic peptide from influenza nucleoprotein, residues 147 to 158. These marker proteins behaved as theoretically expected. However, we observed that several other peptides did not elute in a volume that would have been expected according to their relative molecular mass. Retardation (for example, of somatostatin,  $M_r = 1638$ ) could not be overcome by changes of eluant or column, so that size estimations of small peptides by gel filtration should generally be interpreted with caution.
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  28. Single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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  31. Generation of the H-Y-specific CTL line 11P9: A female B6 mouse was immunized intravenously with  $10^7$  irradiated (33 Gy), male B6 spleen cells. Three weeks later, recipient spleen cells ( $2 \times 10^7$ ) were stimulated *in vitro* against irradiated (33 Gy) male B6 spleen cells ( $2 \times 10^7$ ) in  $\alpha$ -MEM-medium (Gibco) containing 10% fetal bovine serum,  $\beta$ -mercaptoethanol, glutamine, and antibiotics in a 5%  $\text{CO}_2$  atmosphere at 37°C for 7 days. Thereafter, surviving cells were restimulated weekly with irradiated male B6 spleen cells in interleukin-2-supplemented medium.
  32. We thank J. Klein for support; P. Bohley for helpful

discussions; T. Schechinger for help in setting up the HPLC system; H. Kalbacher, K.-H. Wiesmüller, K. Deres, and G. Jung for synthetic peptides; F. Carbone and M. Moore for P13.1 cells; H. Schild for CTL and for reading the manuscript; C.

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## Prevention of HIV-1 Infection and Preservation of CD4 Function by the Binding of CPFs to gp120

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**Infection by human immunodeficiency virus type-1 (HIV-1) is initiated when its envelope protein, gp120, binds to its receptor, the cell surface glycoprotein CD4. Small molecules, termed *N*-carboxymethoxycarbonyl-prolyl-phenylalanyl benzyl esters (CPFs), blocked this binding. CPFs interacted with gp120 and did not interfere with the binding of CD4 to class II major histocompatibility complex molecules. One CPF isomer, CPF(DD), preserved CD4-dependent T cell function while inhibiting HIV-1 infection of H9 tumor cells and human T cells. Although the production of viral proteins in infected T cells is unaltered by CPF(DD), this compound prevents the spread of infection in an *in vitro* model system.**

**C**D4, A SURFACE GLYCOPROTEIN found primarily on a subset of T lymphocytes, is a receptor for both the class II major histocompatibility complex (MHC) antigens (1, 2) and the human immunodeficiency viruses (HIV) (3, 4). CD4 probably binds a monomorphic domain on class II MHC, thereby facilitating antigen recognition and enhancing T cell activation by increasing adhesion and signal transduction (5). HIV binds to CD4 through its envelope glycoprotein, gp120 (4). A COOH-terminal region of gp120 (amino acids 403–421), in particular Trp<sup>411</sup>, has been implicated (6, 7). The most NH<sub>2</sub>-terminal immunoglobulin-like domain of CD4 is sufficient to bind gp120 (8), although the second domain in the intact molecule also contributes to binding (9). The binding sites on CD4 for gp120 and class II MHC overlap (10, 11); however, the binding sites for class II MHC may be more extensive (10, 12). The binding of gp120 blocks CD4 binding to class II MHC and thus inhibits CD4-dependent lymphokine production (2, 13, 14).

Soluble forms of CD4 seem to be promising agents that can inhibit infection by binding to gp120 (15–17), without interfering with the binding of cellular CD4 to the class II MHC proteins (16). However, proteins may have delivery, stability, and expense problems that may not be associated with low molecular weight agents. A mutational analysis of CD4 (8, 11) implicated Phe<sup>43</sup> in the interaction of CD4 with gp120; substitution of Leu for Phe<sup>43</sup> completely abrogated gp120 binding (11). We therefore tested a series of small phenylalanine-containing molecules for inhibition of gp120 binding to CD4. Derivatives of the dipeptide prolylphenylalanine with an NH<sub>2</sub>-terminal carbomethoxycarbonyl moiety and a COOH-terminal benzyl ester (Fig. 1), termed CPFs (*N*-carboxymethoxycarbonyl-prolyl-phenylalanyl benzyl ester), blocked gp120 binding to CD4, reversed the inhibition by gp120 of CD4–class II MHC binding, inhibited infection by HIV, and preserved CD4-dependent lymphokine production in the presence of gp120. By examining a number of closely related compounds we have begun to identify those functional groups responsible for the inhibitory activity. Thus, CPFs are promising forerunners to an effective inhibitor of HIV-1 infection.

The pre-T cell leukemia line HSB-2 was transfected with CD4 and a high expressing clone, HSBCD4-M.23, was isolated (18). Binding of gp120 to CD4 on HSBCD4-M.23 can be detected by flow cytometry after staining with an antiserum to gp120 and a fluoresceinated antibody to immuno-

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globulin. When gp120 is first incubated with CPF(LL) its binding to CD4 is inhibited in a concentration-dependent manner (Fig. 2A). Consistent levels of inhibition of CD4 binding are obtained only when gp120 is incubated first with CPFs. Inhibition is more efficient when the initial incubation is at 37° than at 4°C (19).

The stereochemistry of the CPF peptide backbone appears relatively unimportant. All four possible CPF stereoisomers had similar activities. The stereoisomer containing the (unnatural) D-configuration of both amino acids [CPF(DD)] consistently showed the greatest activity (Fig. 2A). Thus, the amide backbone may not be important for molecular recognition of CPFs; models of CPF(LL) and CPF(DD) indicate that CPF substituents can occupy similar regions of space if the directionality of the amide and NH vectors is ignored (19).

In contrast, the side chains and blocking groups and their relative spacing appear to be critical. Both of the aromatic rings, in the Phe and the COOH-terminal benzyl ester, contribute significantly and approximately equally to the activity of CPF(LL) (Fig. 2B). When either ring is removed, by changing Phe to Ala [CPF(F → A)] or the benzyl ester to a methyl ester [CPF(C-Me)], the inhibitory activity is reduced, with an approximately two- to fivefold greater dose required to achieve 50% inhibition (Fig. 2B). In the region of CD4 identified as a gp120 binding domain, Phe<sup>43</sup> precedes a Leu residue. To test whether the inhibitory activity of CPFs is due to structural homology to CD4, a Leu residue was introduced after the Phe in CPF(LL) [CPF(+Leu)]. The activity of this analog was similar to the aromatic ring deletions, CPF(F → A) and CPF(C-Me). This suggests that extended homology to CD4 may not be important, and that the reduction in activity may be due to the displacement of the benzyl ester relative to the phenyl ring caused by the insertion of the Leu residue.

The NH<sub>2</sub>-terminal carbomethoxycarbonyl moiety appears to be essential to the activity of CPFs. When the dicarbonyl is replaced by a *tert*-butoxycarbonyl [CPF(N-Boc)] moiety, the molecule is inactive (Fig. 2C). This conclusion is further supported by results with a *N*-acetyl analog and the trifluoroacetate salt of the free amine of (D,D)-prolylphenylalanine benzyl ester (19). Changing the position of the NH<sub>2</sub>-terminal carbomethoxycarbonyl group relative to the aromatic rings by deleting the Pro residue [CPF(ΔP)] ablates activity. Likewise, deletion of the Phe residue [CPF(ΔF)] results in inactivity (Fig. 2C).

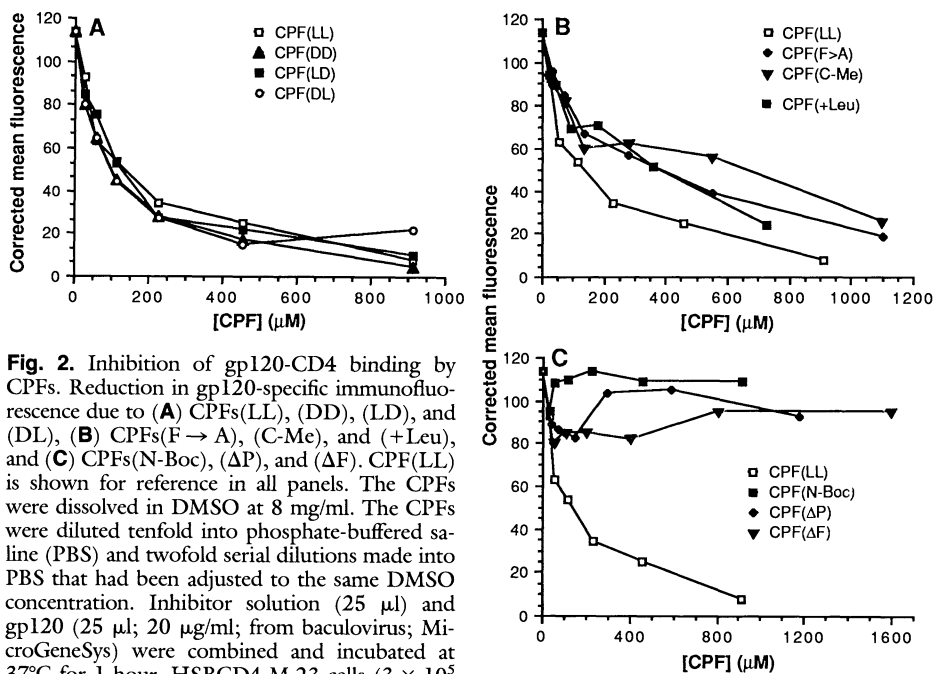
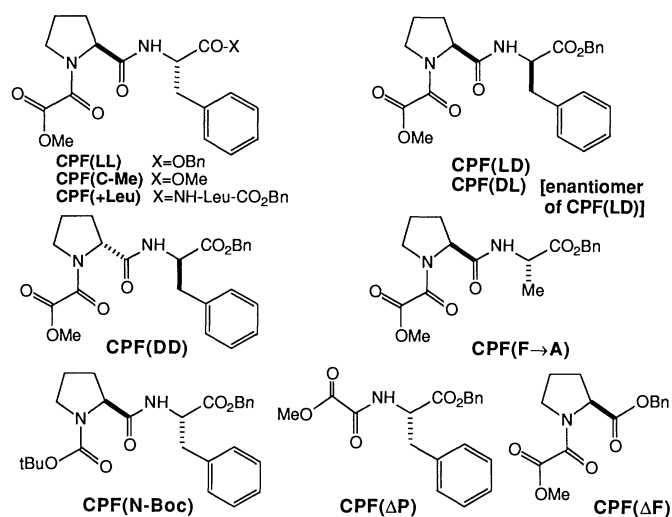
That CPFs bind to gp120 is suggested by the greater inhibition observed when they

are incubated with gp120 before addition to the CD4<sup>+</sup> cells. In addition, an increasing rate of inhibition was observed on increasing the temperature during preincubation (possibly due to the low water solubility of CPFs). Treatment of the HSBCD4-M.23 cell line with CPF(LL) (100 μg/ml) did not affect its staining with the CD4 monoclonal antibodies (MAbs) OKT4, OKT4C,

OKT4D, and Leu3A (19). These last two MAbs block gp120 binding and recognize epitopes that overlap the gp120 binding site (20). This result contrasts with that obtained with aurintricarboxylic acid, which inhibits gp120 binding by interacting with CD4, as detected by staining with MAbs (21).

To demonstrate binding of a CPF to gp120, gp120 was incubated with

**Fig. 1.** Molecular structures of CPFs. The synthesis of CPF(DD) is illustrative of the general synthetic strategy employed in this study: *N*-*tert*-butoxycarbonyl-D-proline (Boc-D-Pro) was coupled to the trifluoroacetic acid (TFA) salt of D-phenylalanine benzyl ester (D-Phe-OBn) by the use of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, hydroxybenzotriazole, and catalytic 4-pyrrolidinopyridine to generate Boc-D-Pro-D-Phe-OBn. TFA-mediated removal of the Boc protecting group and then acylation of the resulting TFA salt of D-Pro-D-Phe-OBn with methyl oxalyl chloride gave CPF(DD).



**Fig. 2.** Inhibition of gp120-CD4 binding by CPFs. Reduction in gp120-specific immunofluorescence due to (A) CPFs(LL), (DD), (LD), and (DL), (B) CPFs(F → A), (C-Me), and (+Leu), and (C) CPFs(N-Boc), (ΔP), and (ΔF). CPF(LL) is shown for reference in all panels. The CPFs were dissolved in DMSO at 8 mg/ml. The CPFs were diluted tenfold into phosphate-buffered saline (PBS) and twofold serial dilutions made into PBS that had been adjusted to the same DMSO concentration. Inhibitor solution (25 μl) and gp120 (25 μl; 20 μg/ml; from baculovirus; MicroGeneSys) were combined and incubated at 37°C for 1 hour. HSBCD4-M.23 cells (3 × 10<sup>5</sup> to 5 × 10<sup>5</sup>) were resuspended with the preincubated gp120-inhibitor mixture and incubated at 4°C for 1 hour. The cells were then pelleted and labeled with rabbit antibodies to gp120 (1:400 in PBS; MicroGeneSys) and then with fluorescein isothiocyanate-conjugated goat antibodies to rabbit immunoglobulin (1:20; Tago). The cells were resuspended with propidium iodide and analyzed by flow cytometry on an Epics V (Coulter) or FACScan (Becton-Dickinson). Values were corrected by subtracting the mean linear fluorescence of cells treated with both antibodies but not exposed to gp120, which did not differ significantly from the intrinsic fluorescence of the cells, and was generally <15. It should be noted that unlike the gp120 used in our previous work (2, 13), 10 μg/ml is a subsaturating concentration for the baculovirus produced gp120 used in these experiments and the relation between fluorescence intensity and gp120 concentration approaches linearity (19). These results are from a single representative experiment. Each compound has been tested in at least three separate experiments.

CPF(DD), extensively diluted, and the gp120 reconcentrated by ultrafiltration. The CPF(DD) was too small [molecular weight (MW) of 438] to be retained by the ultrafiltration membrane (30,000 MW cut-off) (19). Equivalent amounts of gp120 were recovered in the experimental and control retentates, as confirmed by a biuret-type protein microassay (19). The inhibition of the binding to HSB-CD4-M.23 by the CPF(DD)-treated gp120, compared to untreated gp120 or CPF( $\Delta$ F)-treated gp120, was unaffected by dilution and ultrafiltration (19). Since the residual CPF(DD) concentration is too low (diluted more than 20-fold) to effect this degree of inhibition (Fig. 2), it must bind and remain bound to gp120. Similar results were obtained when the dilution and ultrafiltration process was repeated three times (final CPF dilution greater than 8000-fold) (19). These residual CPF concentrations are lower than the  $IC_{50}$  implied by the data in Fig. 2.

The physiologic ligands of CD4 are the class II MHC proteins, which promote conjugate formation in vitro between human CD4<sup>+</sup> murine T cell hybridomas and class II MHC<sup>+</sup> cells (2); gp120 inhibits conjugate formation (2). Conjugate formation of class II MHC<sup>+</sup> cells with HSB-CD4-M.23 cells was inhibited by gp120 and reversed by CPF(DD), but not CPF( $\Delta$ F) (Fig. 3A). There was no inhibition of conjugates with gp120 first incubated with CPF(DD) (19) or with simultaneous addition of all components (Fig. 3A). Addition of CPF(LL) or CPF(DD) restored conjugate formation even when the gp120 had been previously bound to the CD4 (19). The CPFs had no effect on conjugate formation directly (Fig. 3A) and should not interfere with this aspect of T cell function; instead they would be expected to reverse any inhibition due to gp120.

We examined whether CPFs could also restore gp120-inhibited CD4-dependent lymphokine production by CD4<sup>+</sup>, HLA-DR-reactive T cell hybridomas. Gp120 was incubated alone or with CPF(DD) or CPF( $\Delta$ F) and subjected to ultrafiltration to remove excess CPF. The recovered gp120 samples were then incubated with the CD4<sup>+</sup> T cell hybridoma and HLA-DR<sup>+</sup> stimulator (Daudi) cells overnight. The culture supernatants were assayed for interleukin-2 (IL-2) production in a CTLL-20 bioassay. As measured by IL-2 production, CPF(DD) blocked gp120-mediated inhibition of CD4 function, whereas the inactive CPF( $\Delta$ F) did not (Fig. 3B). CPF(DD) alone did not stimulate lymphokine production (22). Similar results were obtained when the ultrafiltration step was deleted (22). These data suggest that although CPF(DD) inhibits

gp120 binding to CD4 it does not affect CD4 participation in T cell activation, an important requirement of therapeutic agents that block receptor-ligand binding.

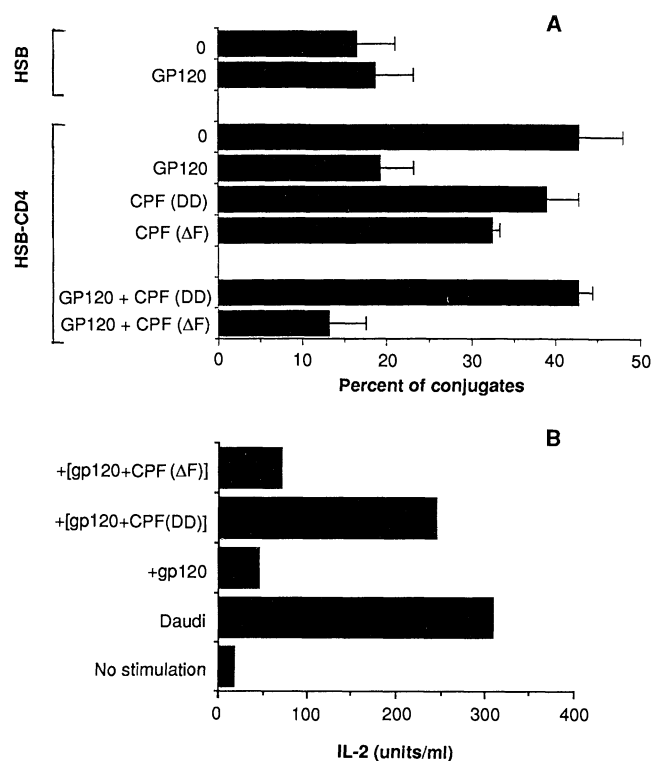
We also examined the effect of CPF(DD) on HIV infection of H9 tumor cells and human T cells. HIV-1 was incubated with CPF(DD) before the addition of virus to the CD4<sup>+</sup> H9 tumor cell. Titration of CPF(DD) revealed that incubation of HIV-1 with CPF(DD) for 1 hour at 37°C at concentrations between 40 and 800  $\mu$ g/ml resulted in increasing inhibition of infection of H9 tumor cells, as measured by production of the HIV-1 p24 antigen (Fig. 4A, HIV-1 strain III<sub>B</sub>; Fig. 4B, strain MN). Similar results were obtained with CD4<sup>+</sup> human peripheral blood T cells, although the concentration of CPF(DD) required to inhibit infectivity was lower than that required to inhibit H9 infection, which may reflect the lower sensitivity of human peripheral blood T cells to HIV-1 infection (Fig. 4C). Incubation of the virus with 160  $\mu$ g/ml of CPF(DD) completely inhibited infection for 30 days, whereas incubation

with 160  $\mu$ g/ml of CPF( $\Delta$ F) showed no inhibition of infectivity. It should also be noted that no concentration of CPF(DD) had any effect on cell viability or growth of H9 cells or human T cells (22).

To probe the viral specificity of CPF(DD), the inhibitor was incubated at 80 or 800  $\mu$ g/ml with coxsackie B3 or echo virus type 1 before addition to HeLa cells. Neither concentration inhibited infection of the HeLa cells by these picornaviruses (22). Thus CPF(DD) possesses viral specificity.

The effect of CPFs on the production of other HIV-1 proteins in infected cells was examined by immunoprecipitating HIV-1-infected H9 cells. Cells infected with HIV that had been previously incubated with CPF(DD) contained no viral proteins; cellular proteins were unaffected (22). In contrast, H9 indicator cells infected with CPF( $\Delta$ F)-exposed virus produced all viral proteins. Identical results were obtained with peripheral blood T cells (22). RNA dot blots revealed an absence of replicating virus in H9 cells incubated with CPF(DD)-treated virus. Addition of CPF(DD) to a cell

**Fig. 3. (A)** Restoration of CD4-class II MHC-mediated conjugate formation by CPFs. Conjugate formation assays were done with the CD4<sup>+</sup> HSB-CD4-M.23 and class II MHC<sup>+</sup> cell lines (Daudi). The HSB-CD4-M.23 cells were labeled with sulfofluorescein diacetate (Molecular Probes, Eugene, Oregon) and Daudi cells with hydroethidine (Polysciences, Inc., Warrington, Pennsylvania) (31). Cells ( $1 \times 10^6$  per milliliter) were mixed at a 1:2 CD4<sup>+</sup> to class II MHC<sup>+</sup> cell ratio and incubated at 37°C for  $\geq 90$  min with CPF (100  $\mu$ g/ml; in DMSO, 1% final concentration), gp120 (20  $\mu$ g/ml), and their mixtures. At the end of the incubation period samples were gently resuspended by repipetting five times through a tip with an internal diameter of 0.5 mm. A conjugate was defined as at least two cells of one color bound to at least one of the other. Blinded samples were run in duplicate and three or more counts of at least 100 fluorescein-labeled cells were done for each sample. The percent of conjugates was calculated as the total number of conjugates divided by the total number of conjugates plus free CD4<sup>+</sup> cells. Results are expressed as the mean value  $\pm$  SEM of the six counts made for each group. **(B)** The inhibition of CD4-dependent IL-2 production by gp120 is blocked by CPF(DD) but not by CPF( $\Delta$ F). Gp120 (5 to 10  $\mu$ g/ml) was incubated for 1 hour at 37°C with or without CPF (200  $\mu$ g/ml). The gp120 solutions were then diluted 20-fold and the gp120 reconcentrated by ultrafiltration in a centricon-30 ultrafiltration apparatus (molecular weight cutoff = 30,000; Amicon). The recovered gp120 solutions were then combined with  $1 \times 10^6$  16CD4-9 T cell hybridoma cells and  $2 \times 10^5$  Daudi cells and cultured overnight at 37°C. IL-2 was detected in the culture supernatants by bioassay (13), except that proliferation was determined colorimetrically as the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). These results are from a single experiment and are representative of at least three separate experiments.



culture already infected with HIV, in contrast, had little effect on viral RNA synthesis (22). Thus CPF(DD) blocks HIV infectivity, but it does not prevent intracellular viral replication.

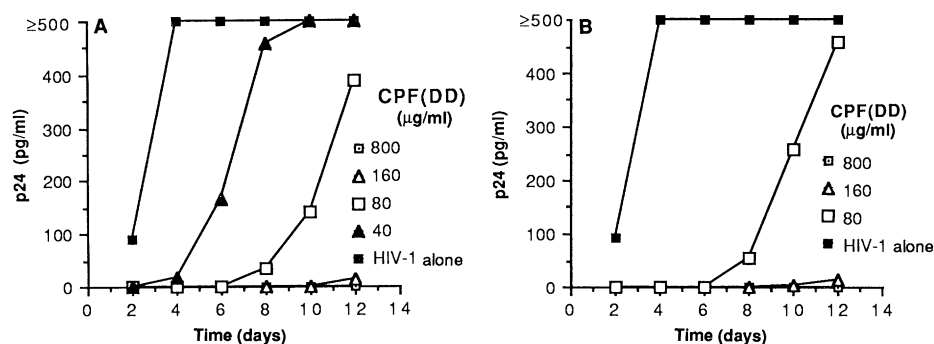
We investigated the ability of CPFs to disrupt the spread of virus from infected to uninfected cells (Fig. 5). Infected peripheral blood T cells ( $10^4$  cells per milliliter) were added to uninfected H9 indicator cells in the presence of CPF(DD) or CPF( $\Delta$ F) (80  $\mu$ g/ml). The T cells produced little virus (as measured by p24 antigen) and died within 3 days after culture, as they were in lymphokine-free medium (22). Incubation of H9 indicator cells with infected T cells for 2, 4, or 6 days resulted in rapid infection of H9 cells with subsequent production of p24 antigen. The addition of CPF( $\Delta$ F) at the time of incubation had no effect on this infection, whereas CPF(DD) completely inhibited infection of the H9 cells (Fig. 5). Therefore, the continuous presence of CPF(DD) inhibits the spread of virus from infected to noninfected cells.

Inhibition of receptor-ligand binding is an attractive antiviral strategy. It acts at the earliest stages in the cycle of infection against a target whose structure is not easily altered in progeny strains without disrupting infectivity. In contrast, it appears that

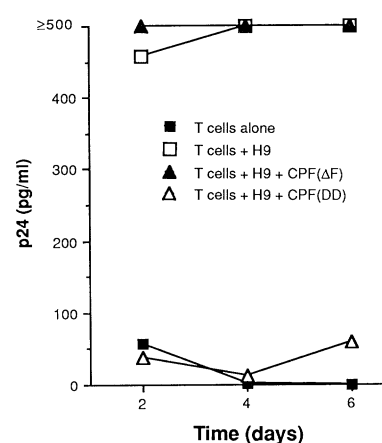
resistance of the virus to enzyme inhibitors can be more readily established without disrupting the target enzyme's essential function. Strains of virus resistant to AZT, the only anti-HIV drug in widespread use, have been detected (23). Because of their different mechanisms of action, CPFs might work in synergy with enzyme inhibitors. High molecular weight inhibitors of receptor-ligand binding such as CD4 analogs, engineered proteins (17), synthetic peptides (24, 25), and dextran sulfate (26) probably require administration by injection to be effective. In contrast, specific antiviral activity of small peptides has been detected in serum after oral administration [for example, see (27)].

Both phenyl rings of the CPFs contribute to the activity and the dicarbonyl moiety appears to be essential. The hydrophobicity of CPFs suggests that they bind to a nonpolar site on gp120, and the contribution of the phenyl rings to their activity may reflect an aromatic stacking interaction in this binding site (28). Replacement of a conserved aromatic residue in the CD4 binding site of gp120, Trp<sup>411</sup>, with nonaromatic amino acids completely abrogates binding (7).

The use of small (di- or tri-) peptides as inhibitors of viral infection is not unprece-



**Fig. 4.** Incubation of virus with CPF(DD) prevents expression of viral proteins in H9 cells and T cells. (A) Increasing amounts of CPF(DD) were incubated with HTLV-III<sub>B</sub> or (B) HTLV-MN (AIDS Research and Reference Reagent Program) at a titer of  $10^6$  tissue culture infectious doses/ml in 1 ml of RPMI 1640 with 10% bovine calf serum for 1 hour prior to their addition to  $10^6$  H9 cells. CPFs were dissolved in DMSO at 8 mg/ml and then diluted into RPMI 1640 and 10% bovine calf serum. Cells were infected by incubation for 1 hour at 37°C. Cells were washed twice and split twice weekly (maintained at a density of approximately  $5 \times 10^5$  cells per milliliter). Supernatants obtained 48 hours after the last split were assessed for the presence of p24 antigen by ELISA (Abbott). (C) Human T cells were obtained by depleting nylon wool passed peripheral blood mononuclear cells by using antibodies to CD8 (OKT8, ATCC) and CD16 (B73.1, gift of G. Trinchieri) followed by antibody-coated magnetic beads (32). Virus was incubated for 1 hour at 37°C with CPF(DD) or CPF( $\Delta$ F). The treated virus preparation was then cultured with cells for 1 hour at 37°C. Cells were then washed twice and maintained in RPMI 1640 with 10% bovine calf serum supplemented with IL-2 (10 units/ml; Collaborative Research). Supernatants were obtained 48 hours after the last split and assayed for p24 antigen. A representative example of three experiments performed is shown.



**Fig. 5.** Continuous exposure to CPF(DD) prevents cell to cell spread of virus. T cells, infected with HIV-1 3 weeks before, were washed extensively and then mixed at a ratio of  $10^4$  T cells to  $5 \times 10^5$  H9 cells in the presence or absence of 80  $\mu$ g/ml of CPF(DD) or CPF( $\Delta$ F) and cultured for 2, 4, or 6 days. Cell densities were adjusted to maintain a concentration of  $5 \times 10^5$  H9 cells per milliliter. ELISA measurements of p24 antigen in the supernatant were made 48 hours after washing the T cell-H9 cell mixture free of virus. One representative experiment (of three performed) is shown.

ented. A series of carbobenzoxy (Z) peptides, including Z-D-Pro-D-Phe, inhibit infection by measles and herpes viruses (27, 29). However, detailed studies of the inhibition of myxoviral infection have indicated that these compounds interact not with a viral protein but with the target cell (30). Thus, CPFs and these peptides employ different mechanisms of action.

The apparent affinity of CPFs for gp120 could in principle be exploited to deliver novel probe or protein-damaging reagents selectively to this target. Such a strategy may offer insights into the location and structure of the CD4 binding site of gp120. CPFs may also represent important candidates for the development of an effective drug for the treatment of AIDS. Clinically, both inhibition of the infection within the individual and reversal of any immunosuppression or toxicity due to soluble gp120 may be expected with the CPFs. The ability of CPF(DD) to prevent the spread of HIV-1 from a small number of afflicted cells to a larger population of uninfected cells is of considerable therapeutic importance. This attribute is required of any agent designed to block the dissemination of an existing HIV-1 infection.

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## Genetic Differences in the Ethanol Sensitivity of GABA<sub>A</sub> Receptors Expressed in *Xenopus* Oocytes

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Animal lines selected for differences in drug sensitivity can be used to help determine the molecular basis of drug action. Long-sleep (LS) and short-sleep (SS) mice differ markedly in their genetic sensitivity to ethanol. To investigate the molecular basis for this difference, mRNA from brains of LS and SS mice was expressed in *Xenopus* oocytes and the ethanol sensitivity of gamma-aminobutyric acid A (GABA<sub>A</sub>)- and N-methyl D-aspartate (NMDA)-activated ion channels was tested. Ethanol facilitated GABA responses in oocytes injected with mRNA from LS mice but antagonized responses in oocytes injected with mRNA from SS animals. Ethanol inhibited NMDA responses equally in the two lines. Thus, genes coding for the GABA<sub>A</sub> receptor or associated proteins may be critical determinants of individual differences in ethanol sensitivity.

INDIVIDUAL HUMANS AND RODENTS differ markedly in their behavioral sensitivity to ethanol (1). Behavioral sensitivity has also been used as a marker or index for selection of a number of different rodent lines (2, 3). The LS and SS mouse lines (2) were selected for differential sensitivity to the acute hypnotic effect of ethanol determined by the duration of loss of the righting reflex ("sleep time") after injection of ethanol. The lines differ markedly in this respect. Behavioral and biochemical studies on LS and SS mice indicate that they have similar ethanol metabolism and pharmacokinetics. However, the two lines differ in the response of neuronal GABA receptors to ethanol (4) as well as in the sensitivity of the GABA receptor to benzodiazepines (5), sug-

gesting that the genetic differences seen in response to ethanol may be the result of modifications of the GABA<sub>A</sub> receptor-chloride channel complex. This complex is composed of multiple subunits, many of which have been cloned, sequenced, and expressed in *Xenopus* oocytes or transfected cells (6, 7). Thus, there are many subunits, and probably different subtypes, of GABA<sub>A</sub> receptors that could underlie genetic differences in drug action.

The similarity between the actions of ethanol and sedative drugs such as benzodiazepines and barbiturates that enhance GABA action, as well as behavioral studies with GABAergic drugs, provides evidence that ethanol exerts at least some of its effects by enhancing the function of the GABA receptor (8). More directly, biochemical techniques with radioactive Cl<sup>-</sup> show a potentiating effect of intoxicating concentrations of ethanol on functional GABA-activated Cl<sup>-</sup> channels in brain membranes and in cul-

tured spinal cord neurons (4, 9); electrophysiological studies are less consistent with regard to ethanol-GABA receptor interactions (10). GABA<sub>A</sub> receptor subtypes or different membrane environments may account for these diverse results.

Our approach to defining differences in the GABA<sub>A</sub> receptor-Cl<sup>-</sup> channel complex that might underlie the genetic differences in ethanol susceptibility was to prepare mRNA from LS and SS mouse whole brain and express this mRNA in *Xenopus* oocytes (7, 11). Receptor function was studied electrophysiologically with voltage-clamp techniques (11). We observed inward currents in response to application of GABA when the oocyte membrane was voltage-clamped between -60 and -100 mV, which is consistent with the activation of a Cl<sup>-</sup> conductance (the reversal potential for Cl<sup>-</sup> is approximately -20 mV in the oocytes) (Fig. 1). Maximum currents observed with 100 μM GABA were 147 ± 30 nA and 163 ± 52 nA (mean ± SEM, n = 30 for each group) for LS and SS GABA receptors, respectively. GABA was used at 10 or 30 μM (20 to 50% of the maximum response) in combination with modulatory drugs. These submaximal concentrations also produced currents that were not statistically different between oocytes injected with LS or SS mRNA (n = 7 to 15). The receptors that were expressed showed pharmacological responses typical of native GABA<sub>A</sub> receptors; they were facilitated by pentobarbital and diazepam and inhibited by picrotoxinin (Fig. 1A). Exposure of oocytes to ethanol alone at concentrations less than 100 mM did not elicit any current response.

Application of 10 to 50 mM ethanol together with GABA to oocytes expressing

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