0026-895X/01/6002-341–347$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:341–347, 2001

Vol. 60, No. 2
721/921112
Printed in U.S.A.

A Peptide Derived from Bee Venom-Secreted Phospholipase A_2 Inhibits Replication of T-Cell Tropic HIV-1 Strains via Interaction with the CXCR4 Chemokine Receptor

DAVID FENARD, GERARD LAMBEAU, THOMAS MAURIN, JEAN-CLAUDE LEFEBVRE, and ALAIN DOGLIO

Laboratoire de Virologie, Institut National de la Sante et de la Recherche Medicale U526, Faculté de Médecine, Nice, France (D.F., T.M., J.C.L., A.D.); and Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique UPR411, Valbonne, France (G.L.)

Received November 28, 2000; accepted May 4, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

We have previously shown that secreted phospholipases A_2 ($sPLA_2$) from bee and snake venoms have potent anti-human immunodeficiency virus (HIV) activity (Fenard et al., 1999). These $sPLA_2$s block HIV-1 entry into host cells through a mechanism linked to $sPLA_2$ binding to cells. In this study, 12 synthetic peptides derived from bee venom $sPLA_2$ ($bvPLA_2$) have been tested for inhibition of HIV-1 infection. The p3bv peptide (amino acids 21 to 35 of $bvPLA_2$) was found to inhibit the replication of T-lymphotropic (T-tropic) HIV-1 isolates (ID_{50} = 2 μM) but was without effect on monocytotropic (M-tropic) HIV-1 isolates. p3bv was also found capable of preventing the cell-cell fusion process mediated by T-tropic HIV-1 envelope. Finally, p3bv can inhibit the binding of radiolabeled stromal cell-derived factor (SDF)-1α, the natural ligand of CXCR4, and the binding of 12G5, an anti-CXCR4 monoclonal antibody. Taken together, these results indicate that p3bv blocks the replication of T-tropic HIV-1 strains by interacting with CXCR4. Its mechanism of action however appears distinct from that of $bvPLA_2$ because the latter inhibits replication of both T-tropic and M-tropic isolates and does not compete with SDF-1α and 12G5 binding to CXCR4.

HIV-1 isolates are known to display distinct cellular tropism. Most primary HIV-1 isolates, referred to as "M-tropic" HIV-1 isolates, can infect human macrophages and primary T cells. On the other hand, laboratory-adapted HIV-1 isolates, known as "T-tropic", can infect primary and immortalized human T cells. Besides binding to the CD4 receptor (Dalgleish et al., 1984), this tropism is determined at the level of virus entry by the use of a specific coreceptor that belongs to the chemokine receptor family (Chan and Kim, 1998; Berger et al., 1999; Ross et al., 1999). The CC chemokine receptor (CCR) 5 is the major coreceptor for M-tropic HIV-1 strains, now referred to as R5 viruses (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996). On the other hand, the CXC chemokine receptor (CXCR) 4 is the major coreceptor for T-tropic HIV-1 strains, referred to as X4 viruses (Feng et al., 1996). To a lesser extent, other members of the chemokine receptor family, like CCR3, CCR2b, CCR8, are also capable of mediating HIV entry into host cells (Berger et al., 1999; Ross et al., 1999). The chemokines and their derivatives thus form a potent class of HIV inhibitors and could be potential therapeutic agents (Howard et al., 1999). These factors are able to compete with HIV-1 envelope glycoprotein (gp120) for binding to the chemokine receptor and can also down-regulate their cognate receptor (Chan and Kim, 1998; Berger et al., 1999; Ross et al., 1999). For instance, the CXC chemokine SDF-1α, which binds to CXCR4, specifically blocks the replication of X4 viruses (Bleul et al., 1996; Oberlin et al., 1996). Furthermore, various low-molecular weight compounds that bind to CXCR4 and inhibit HIV-1 replication have been identified. They include positively charged peptides (Doranz et al., 1997), the bicyclam compound AMD3100 (Schols et al., 1997), and T22, a strong anti-HIV peptide (Murakami et al., 1997b).

Secreted phospholipases A_2 ($sPLA_2$) form a structurally related family of enzymes that catalyze the hydrolysis of glycerophospholipids to produce free fatty acids and lyso-

This work was supported by the Centre National de la Recherche Scientifique, the BV Foundation Limited, the Association pour la Recherche sur le Cancer, and the Ministère de la Défense Nationale (Grant DGA-DRET 96/096). D. Fenard is a recipient of a grant from Sidaction (Paris, France).

ABBREVIATIONS: HIV, human immunodeficiency virus; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; gp120, HIV-1 envelope glycoprotein; pCa.r, reduced form of pCa; $sPLA_2$, secreted phospholipase A_2; $bvPLA_2$, bee venom $sPLA_2$; AIDS, acquired immunodeficiency syndrome; mAb, monoclonal antibody; β-gal, β-galactosidase; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; SDF, stromal cell-derived factor; T-tropic, T-lymphotropic; M-tropic, monocytotropic.

phospholipids (Gelb et al., 1995; Dennis, 1997; Murakami et al., 1997a; Tischfield, 1997; Lambeau and Lazdunski, 1999; Valentin and Lambeau, 2000). A diversity of $sPLA_2$s has been purified from snake and insect venoms, and these enzymes have been shown to exert a wide array of pharmacological effects, including neurotoxicity and myotoxicity (Kini and Evans, 1989). Recent studies have revealed that a diversity of $sPLA_2$s is also present in mammals (Cupillard et al., 1997; Ishizaki et al., 1999; Valentin et al., 1999a, 1999b, 2000; Gelb et al., 2000; Suzuki et al., 2000; Valentin and Lambeau, 2000), and these $sPLA_2$s are likely to be associated with various physiological and pathological processes, including host defense (Murakami et al., 1997a; Tischfield, 1997; Lambeau and Lazdunski, 1999; Valentin and Lambeau, 2000). Furthermore, specific receptors for $sPLA_2$s have been identified suggesting that these enzymes may also function as ligands (Lambeau and Lazdunski, 1999).

We have recently shown that several venom $sPLA_2$s are potent inhibitors of the replication of HIV-1 and HIV-2 (Fenard et al., 1999). For instance, bee venom $sPLA_2$ ($bvPLA_2$) is able to block the entry of both X4 and R5 viruses into host cells at nanomolar concentrations (Fenard et al., 1999). The inhibition does not result from a virucidal or cytotoxic effect but involves a more specific mechanism linked to $sPLA_2$ binding to cells (Fenard et al., 1999). In this study, we have analyzed the inhibitory effect of several synthetic peptides derived from $bvPLA_2$ on HIV-1 replication. A peptide of 15 amino acids that corresponds to a surface-exposed loop of $bvPLA_2$ was found to display a potent anti-HIV-1 activity against T-tropic HIV-1 strains and can bind to CXCR4, the major coreceptor of these T-tropic HIV-1 strains.

Materials and Methods

Plasmids and Reagents. Plasmids for HIV-1 (pNL.AD8, pYU2, pHXB2, pBRU-2) and pCMVtat were kindly provided by Drs. P. Charneau and N. Israel, respectively (Pasteur Institute, Paris, France). pHXB2-env was obtained from Drs. Kathleen Page and Dan Littman through the National Institutes of Health AIDS Research & Reference Reagent Program (Rockville, MD). Peptides were synthesized by the Neosystem Laboratory (Strasbourg, France). pCa was synthesized and oxidized to allow the formation of a disulfide bond between cysteines 9 and 31. The 12G5 anti-CXCR4 monoclonal antibody (mAb) was obtained from Dr. James Hoxie (NIH AIDS Research & Reference Reagent Program), 6H8 was from Dr. F. Arenzana-Deisdedos (Institut Pasteur), and anti-human human leukocyte antigen class I mAb (W6/32) was from DAKO (Glostrup, Denmark). Human recombinant SDF-1α was from PeproTech, Inc. (Rocky Hill, UK), and $bvPLA_2$ was prepared as described (Lambeau et al., 1989).

Cell Culture. P4 cells are HeLa $CD4^+$ cells in which transactivation by HIV-1 Tat protein induces expression of the *Escherichia coli Lac Z* gene from the HIV-1 long terminal repeat (Charneau et al., 1994). P4-CCR5 cells are P4 cells expressing the CCR5 receptor (Fenard et al., 1999). These cells and the human embryonic kidney 293 cells (ATCC, CRL-1573) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 7% fetal bovine serum (Biomedia, Boussens, France). CEM is a $CD4^+$ T-cell line obtained from NIH AIDS Research & Reference Reagent Program. These cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum.

HIV-1 Infection Assays. $HIV\text{-}1_{ADA}$, $HIV\text{-}1_{YU2}$, $HIV\text{-}1_{HXB2}$, and $HIV\text{-}1_{BRU}$ were produced after transfection of the respective plasmids pNL.AD8, pYU2, pHXB2, and pBRU-2 in 293 cells using a Ca^{2+} phosphate mammalian transfection kit (Stratagene, Strasbourg, France). Three days after transfection, supernatants were collected, centrifuged (1500*g*, 15 min), and stored at −80°C. High-titer viral stocks of $HIV\text{-}1_{BRU}$ were prepared as previously described (Fenard et al., 1999). Viral stocks were evaluated for HIV-1 viral capsid protein content using an enzyme-linked immunosorbent assay kit (Organon Teknica, Fresnel, France). Single rounds of viral replication were performed as previously described (Fenard et al., 1999). Briefly, P4-CCR5 cells, seeded in 24-well plates, were infected with HIV-1 (100 ng of p24) in the presence or absence of various effectors for 8 h, then the incubation medium was replaced with fresh medium containing 50 μM 3′-azido-3′-deoxythymidine (AZT; Sigma-Aldrich, St. Quentin Fallavier, France). Two days later, cells were lysed, and 98 β-galactosidase (β-gal) activity was measured and used as an index of HIV-1 replication. For syncytium formation assay, 293 cells were cotransfected with pHXB2-env and pCMVtat and mixed 1 day later with P4 cells (3:1 ratio) in the presence or absence of effectors. After 48 h of coculture, cells were lysed and the level of β-gal activity was used as an index of cell-cell fusion. Time-of-addition experiments of p3bv were performed as previously described (Fenard et al., 1999). Briefly, p3bv was added at different times before or after infection of P4-CCR5 cells with $HIV\text{-}1_{BRU}$ (up to 8 h), and cells were then processed as described above for single round infection assay.

Binding Experiments. SDF-1α-binding experiments were performed in 500 μl of binding buffer (RPMI 1640 medium, 0.1% bovine serum albumin) containing 2×10^5 CEM cells, 50 pM ^{125}I-SDF-1α (PerkinElmer Life Science Products, Courtaboeuf, France), and various concentrations of unlabeled SDF-1α or peptides. After 1 h at 25°C, incubations were layered on 500 μl of fetal bovine serum, centrifuged for 3 min at 10,000*g*, and analyzed for radioactivity associated with cell pellets. Nonspecific ^{125}I-SDF-1α binding was determined in the presence of 200 nM unlabeled SDF-1α. For cytometric analysis, 10^6 CEM cells were incubated in 200 μl of phosphate-buffered saline at 4°C for 30 min in the presence or absence of effectors, and mAbs (5 μg/ml) were then added for another 30-min incubation period. After two washes, cells were incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (DAKO) for 30 min. Cells were washed, resuspended in phosphate-buffered saline and 4% formaldehyde, and analyzed for fluorescence using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). Binding competition studies with radiolabeled $sPLA_2$s were performed as previously described (Fenard et al., 1999).

Down-Regulation Experiments. Experiments were performed as previously described (Tilton et al., 2000). Briefly, CEM cells (10^6 cells/ml) were incubated at 37°C in RPMI 1640 medium supplemented with 5% fetal calf serum in the presence of SDF-1α (10 nM) or p3bv (20 μM). At different times after the addition of effectors, cells were chilled on ice and stained with mAbs against CXCR4 (6H8 and 12G5 mAbs) or isotype-matched control IgG, followed by FITC-conjugated goat anti-mouse IgG. Cell-associated fluorescence was analyzed with a FACScan flow cytometer.

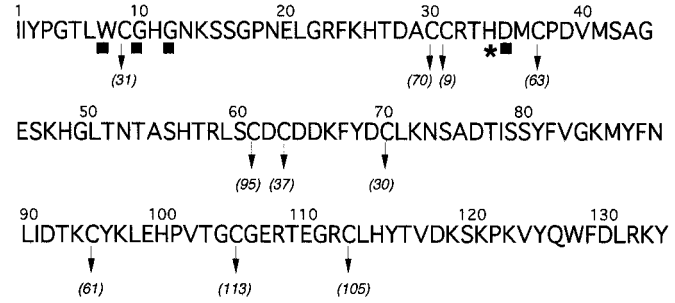
Results

Antiviral Activities of $bvPLA_2$-Derived Peptides. The three-dimensional crystal structure of $bvPLA_2$ has been obtained at a very high resolution, and this structure reveals several domains of homology with other $sPLA_2$s and a rigid network of disulfide bonds that stabilizes the tertiary structure (Scott et al., 1990; Fig. 1A). Twelve overlapping synthetic peptides covering the entire $bvPLA_2$ sequence were first designed (Fig. 1B) and analyzed for their antiviral activity in a single round infection assay using P4-CCR5 cells infected with R5 ($HIV\text{-}1_{ADA}$) or X4 ($HIV\text{-}1_{BRU}$) HIV-1 viruses. Among these peptides, the p3bv peptide (residues 21–35) was found to have antiviral activity against $HIV\text{-}1_{BRU}$ but was without effect on $HIV\text{-}1_{ADA}$ replication (Fig. 1B). The effect of

p3bv appears specific because all other bvPLA$_2$ peptides were without significant anti-HIV-1 activity (Fig. 1B). As shown in Fig. 2A, the ID$_{50}$ value of X4 HIV-1 virus replication (HIV-1$_{BRU}$ and HIV-1$_{HXB2}$) for p3bv concentrations is close to 2 μM, and replication of these X4 viruses is fully inhibited above 10 μM. The lack of effect of p3bv on the replication of R5 HIV-1 viruses was also confirmed using HIV-1$_{ADA}$ and HIV-1$_{YU2}$ (Fig. 2A).

A second set of peptides related to p3bv was then designed (Fig. 3A). Since p3bv contains two cysteines, this peptide possibly forms a dimer, and thereby interferes with HIV-1 replication. To address this possibility, we designed a new peptide called p3bvS in which the two cysteines are replaced by serines. This peptide has the same antiviral activity as p3bv (Fig. 3B), indicating that p3bv presumably acts as a monomer to inhibit HIV-1 replication. We next designed three small peptides of nine residues, corresponding to the N-terminal (p3bv1), middle (p3bv2), and C-terminal (p3bv3) part of p3bv (Fig. 3A). Of these three peptides, p3bv1 that contains the N-terminal sequence of p3bv (residues 21–29) displays the strongest antiviral activity (ID$_{50}$ = 8 μM, data not shown), indicating that the N-terminal sequence of p3bv is crucially involved in antiviral activity (Fig. 3B). Since the N-terminal sequence of p3bv is in close vicinity to the Ca^{2+}-binding loop of bvPLA$_2$ (Scott et al., 1990), we designed a larger peptide called pCa (residues 6–35; Fig. 3A) that, in addition to p3bv, contains residues forming the Ca^{2+}-binding domain of bvPLA$_2$ (Scott et al., 1990). Furthermore, since the Ca^{2+}-binding loop of bvPLA$_2$ contains a disulfide bond that links the cysteines 9 and 31 (Fig. 1A), we synthesized both oxidized (pCa) and reduced (pCa.r) forms of this peptide and assayed them for HIV-1 inhibition. As shown in Fig. 3B, only pCa.r is able to inhibit HIV-1$_{BRU}$ replication, indicating that pCa has to be reduced to acquire an active conformation. pCa.r was found to inhibit the viral replication with an ID$_{50}$ value of 4 μM (data not shown). This

A



B

peptide name	amino acid position	% of infection HIV$_{ADA}$	HIV$_{BRU}$
p1bv	1-15	120	102
p2bv	11-25	105	98
p3bv	21-35	150	2
p4bv	31-46	110	100
p5bv	41-55	110	78
p6bv	51-99	75	90
p7bv	61-99	98	90
p8bv	81-99	150	96
p9bv	91-105	100	92
p10bv	101-115	90	70
p11bv	111-125	98	83
p12bv	121-134	106	95

Fig. 1. Inhibition of HIV-1 replication by various synthetic peptides derived from bvPLA$_2$. A, amino acid sequence of bvPLA$_2$ (Scott et al., 1990). Numbers in parentheses indicate the positions of cysteines involved in disulfide bonds. *, active site histidine; ■, residues involved in the coordination of Ca^{2+} cofactor. B, P4-CCR5 cells were infected for a single round of viral replication with HIV-1$_{BRU}$ or HIV-1$_{ADA}$ in the absence or presence of the different synthetic peptides (20 μM). The level of viral replication was determined 2 days later by β-gal assay. Maximal viral replication (100%) was measured in the absence of any peptide. Each value represents the mean of at least three independent experiments with S.D. less than 10%.

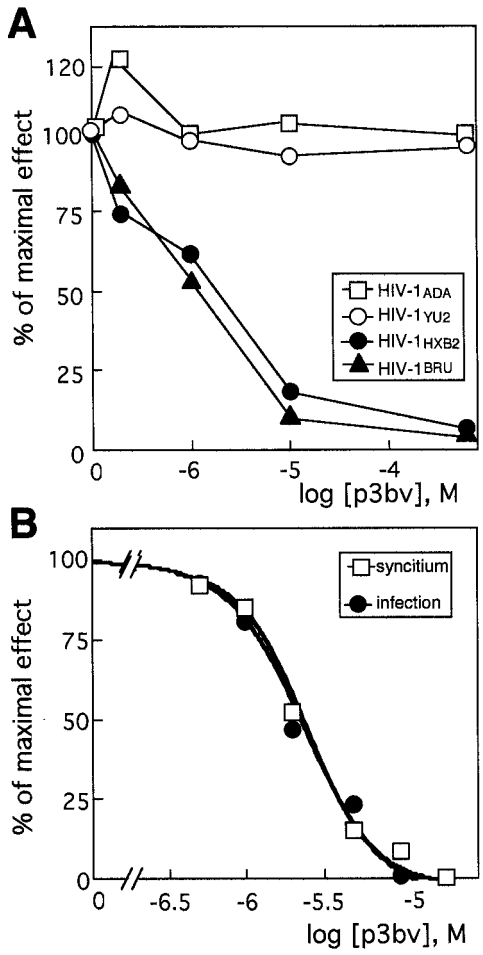


Fig. 2. Effects of p3bv on M- and T-tropic HIV-1 infections (A) and cell-cell fusion (B). A, P4-CCR5 cells were infected with different HIV-1 strains in the presence of different concentrations of p3bv. HIV-1$_{ADA}$ and HIV-1$_{YU2}$ were used as prototypes of M-tropic HIV-1 strains (R5 viruses), HIV-1$_{BRU}$ and HIV-1$_{HXB2}$ were used as prototypes of T-tropic HIV-1 strains (X4 viruses). The level of viral replication was determined 2 days later by β-gal assay. Maximal viral replication (100%) was measured in the absence of peptide. B, the syncytia formation was analyzed using 293 cells cotransfected with the plasmids coding for HIV-1$_{HXB2}$ gp120 and HIV-1 Tat protein. The next day, the 293-transfected cells were mixed with P4 cells in the absence or presence of various concentrations of the p3bv peptide, and the level of cell-cell fusion was estimated 48 h later by a β-gal assay, as described under *Materials and Methods*. Results are representative of two independent experiments with S.D. less than 10%.

value is very similar to that measured for p3bv, suggesting that the 15 N-terminal residues composing the Ca^{2+}-loop of $bvPLA_2$ do not play a critical role in the antiviral activity.

p3bv Prevents Cell-Cell Fusion and Acts Early during the Viral Cycle. The possible effect of p3bv on the inhibition of fusion between viral and cellular membranes was evaluated using syncytium formation assay. As shown in Fig. 2B, p3bv inhibits the cell-cell fusion process between 293 cells expressing a viral envelope protein from an X4 virus ($HIV\text{-}1_{HXB2}$) and P4 cells. Interestingly, the concentrations of p3bv that inhibit the fusion process are very similar to those required for inhibition of infection, both phenomena showing identical ID_{50} values of 2 μM (Fig. 2B). Conversely, p8bv, devoid of antiviral activity (Fig. 1B), is unable to prevent the formation of syncytia (data not shown), indicating a specific effect of p3bv on the cell-cell fusion process. This result also indicates that p3bv may act in an early step during the viral cycle by preventing the fusion between viral and cell membranes. This view was confirmed by time-of-addition experiments indicating that p3bv acts early during the HIV-1 cycle with a half-time effect of about 1.5 h (data not shown).

Inhibition of SDF-1α and 12G5 mAb Binding to CXCR4 by p3bv. Since p3bv specifically inhibits the replication of X4 viruses and yet has no effect on the replication of R5 viruses, it was possible that this peptide interacts with CXCR4, the major coreceptor of X4 viruses. This possibility was addressed by first analyzing the inhibitory effect of p3bv on the binding of ^{125}I-SDF-1α to CXCR4. Specific SDF-1α binding was observed in CEM cells, and unlabeled SDF-1α was found to inhibit the binding of ^{125}I-SDF-1α with a high affinity of 6 nM (Fig. 4A), similar to that previously measured for the interaction of ^{125}I-SDF-1α to the CXCR4 receptor (Crump et al., 1997). Interestingly, p3bv was also able to inhibit the binding of ^{125}I-SDF-1α with a $K_{0.5}$ value close to 2

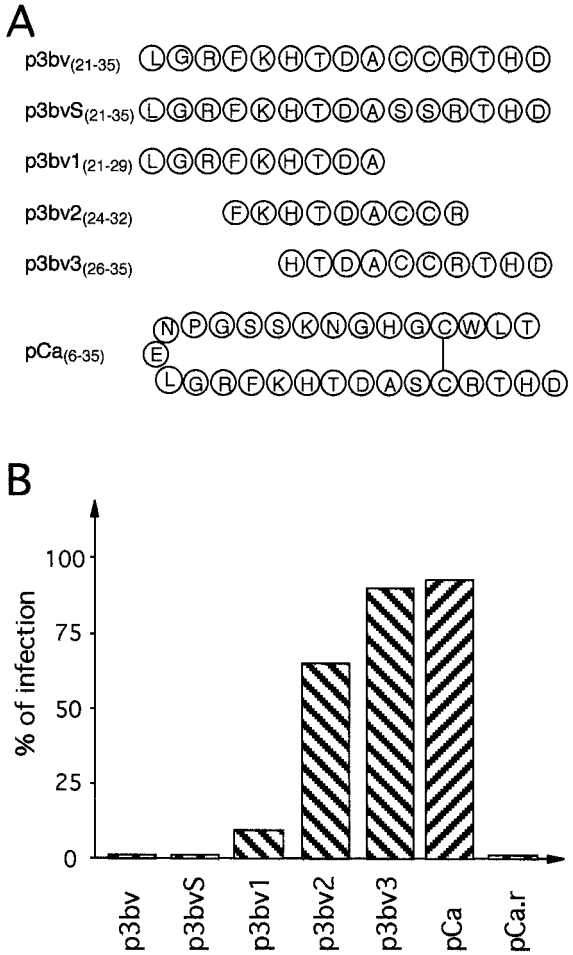


Fig. 3. Antiviral effects of the peptides derived from p3bv. A, amino acid sequence and position of the different peptides. The disulfide bond between cysteines 9 and 31 in pCa peptide is indicated by a plain. Numbers in parentheses indicate the position of each peptide within the $bvPLA_2$ sequence. B, anti-HIV-1 effects of the peptides shown in A. P4-CCR5 cells were infected with $HIV\text{-}1_{BRU}$ in the absence or presence of peptide (20 μM) for a single round of replication as indicated in the legend to Fig. 1. pCa.r was obtained after a treatment at 56°C during 30 min in the presence of 50 mM dithiothreitol. Each value represents the mean of at least three independent experiments with S.D. less than 10%.

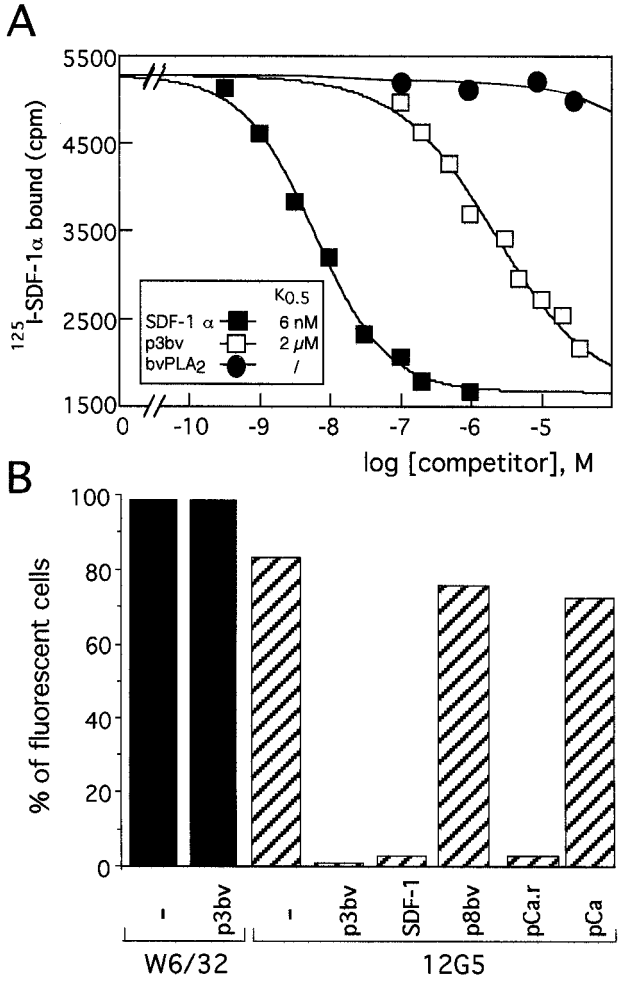


Fig. 4. Effects of p3bv and $bvPLA_2$ on binding of ^{125}I-SDF-1α and 12G5 mAb to CXCR4. A, inhibition of ^{125}I-SDF-1α binding to CEM cells by p3bv and $bvPLA_2$. CEM cells (4×10^5 cells/ml) were incubated with iodinated SDF-1α (50 pM) in the absence or presence of various concentrations of unlabeled SDF-1α, p3bv, or $bvPLA_2$. After 1 h, incubations were layered onto a cushion of fetal bovine serum, centrifuged, and the radioactivity associated with cellular pellets was counted. B, inhibition of 12G5 binding to CXCR4. CEM cells (5×10^6 cells/ml) were incubated for 45 min at 4°C in the absence or presence of each peptide (20 μM) or SDF-1 (250 nM). The monoclonal antibodies 12G5 or W6/32 (5 μg/ml) were then added for 30 min. After several washes, the binding of antibodies was detected using an FITC-conjugated secondary antibody. The percentage of positive cells was determined using a FACS scan analyzer. Results are representative of three different experiments with S.D. less than 10%.

μM (Fig. 4A), indicating that p3bv binds to CXCR4 receptors. This result appeared in marked contrast with bvPLA$_2$, which was unable to inhibit the binding of ^{125}I-SDF-1α (Fig. 4A) even at concentrations up to 10 μM. Furthermore, competition binding experiments between radiolabeled sPLA$_2$ (Fenard et al., 1999) and unlabeled peptide (up to 10 μM) indicate that p3bv does not compete with sPLA$_2$ binding (data not shown). This result suggests that the binding sites of p3bv and sPLA$_2$ are distinct. To further demonstrate that p3bv binds to CXCR4, we analyzed the effects of this peptide on the binding of 12G5, a mAb specific for CXCR4. The binding of 12G5, as measured by FACS analysis, was completely inhibited by p3bv (Fig. 4B), confirming that p3bv binds to CXCR4 receptors. The effect of p3bv on 12G5 antibody is specific, since it does not prevent the binding of W6/32, a mAb known to recognize the human leukocyte antigen class I surface antigen (Fig. 4B). Finally, the binding of 12G5 is also inhibited by pCa.r, which has antiviral activity, but not by the oxidized form of pCa and by the p8bv peptide, which has no effect on HIV-1 infection (Figs. 3 and 4B). A possible effect of p3bv on the down-regulation of CXCR4 was analyzed by flow cytometry using two mAbs (6H8 and 12G5) that recognize different surface-exposed epitopes of CXCR4. 6H8 mAb is known to be specific for the N terminus of CXCR4 (Tilton et al., 2000) and shows only borderline interference with SDF-1α or p3bv binding (Fig. 5; Tilton et al. 2000). On the other hand, both ligands compete with 12G5 mAb. As shown in Fig. 5, p3bv does not induce CXCR4 down-regulation since, in the presence of a saturating amount of this peptide, the surface expression of CXCR4 is not modified when detected with 6H8 mAb. On the contrary, SDF-1 promotes a rapid down-regulation of CXCR4 (Fig. 5). Taken together, these results suggest that the p3bv peptide specifically interacts with CXCR4, but does not promote its down-regulation, and that this interaction is responsible for the inhibition of X4 HIV-1 strains.

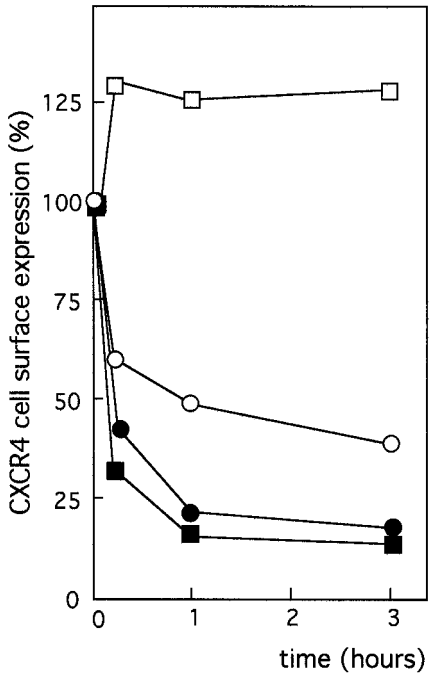


Fig. 5. Effect of p3bv on the down-regulation of CXCR4. CEM cells were incubated at 37°C in RPMI 1640 medium supplemented with 5% fetal calf serum in the presence of 10 nM SDF-1α (filled symbols) or 20 μM p3bv (open symbols) for the indicated times. Cell surface-exposed CXCR4 was detected with mAb 12G5 (circles) or mAb 6H8 (squares). mAb 6H8, but not mAb 12G5, recognizes the NH_2 terminus of CXCR4 and shows only borderline interference with SDF-1α binding. Cells were then stained using FITC-conjugated goat anti-mouse IgG and detected with a FACScan flow cytometer. The percentage of CXCR4 surface expression was calculated from the relative fluorescence intensity. Results are representative of at least three independent determinations.

Discussion

Various peptides and small molecules have been previously described for their ability to block HIV replication through binding to CXCR4 (Doranz et al., 1997; Murakami et al., 1997b; Schols et al., 1997). These molecules act by inhibiting the binding of gp120 to CXCR4 or by down-regulating the expression of CXCR4. We describe here a novel peptide called p3bv, which inhibits HIV-1 replication through binding to CXCR4 with an ID_{50} value close to 2 μM. Furthermore, we show that p3bv does not induce down-regulation of CXCR4. This result indicates that p3bv probably blocks HIV-1 replication by preventing the interaction between CXCR4 and viral components (i.e., the HIV-1 envelope protein).

The p3bv peptide is derived from bvPLA$_2$, which was previously described as a very potent inhibitor of replication of various HIV-1 isolates (Fenard et al., 1999). However, p3bv appears as a weaker inhibitor than bvPLA$_2$, since the ID_{50} value of bvPLA$_2$ is close to 0.6 nM (Fenard et al., 1999). The relatively weak ID_{50} value of p3bv does not appear to result from rapid uptake or degradation of the peptide, since pretreatment of cells with p3bv for up to 6 h before virus addition results in similar levels of HIV-1 inhibition compared with experiments performed without pretreatment (data not shown). This result also confirms that p3bv does not induce down-regulation of CXCR4.

Unexpectedly, we found that p3bv behaves differently than bvPLA$_2$. Indeed, p3bv can only inhibit the replication of X4 viruses, whereas bvPLA$_2$ is able to inhibit both X4 and R5 viruses (Fenard et al., 1999). Furthermore, p3bv inhibits cell-cell fusion (Fig. 2), whereas bvPLA$_2$ was previously shown to be unable to inhibit cell-cell fusion under similar experimental conditions (Fenard et al., 1999). Finally, as monitored by the interaction of SDF-1α and 12G5 antibody with CXCR4, p3bv appears to be a ligand for CXCR4 (Fig. 4). This result appears in marked contrast with bvPLA$_2$, which is unable to inhibit the binding of SDF-1α (Fig. 4A) and 12G5 (data not shown) even at high concentrations up to 10 μM. Taken together, these differences suggest that the mechanisms of HIV inhibition by bvPLA$_2$ and p3bv are distinct. Alternatively, these discrepancies suggest that the mechanism of HIV-1 inhibition by bvPLA$_2$ is a complex process involving multiple steps that cannot be solely explained by the binding of p3bv to the CXCR4 coreceptor.

In our previous study, we have shown that the mechanism of antiviral activity by bvPLA$_2$ is presumably linked to a high-affinity binding of the sPLA$_2$ to host cells ($K_{0,5}$ = 0.08 nM) (Fenard et al., 1999). Our above results indicate that bvPLA$_2$ has no affinity for CXCR4, and thus this receptor is probably not the primary cellular target of bvPLA$_2$. CXCR4, however, appears to bind p3bv derived from bvPLA$_2$, and this binding is most likely explained by the HIV-1 inhibitory

effect of this peptide. This suggests that within the bvPLA$_2$, the p3bv region does not have the same conformation as that of the synthetic p3bv peptide. This hypothesis is supported in part by the fact that the reduced, but not oxidized, form of pCa inhibits HIV-1 infection and binding to CXCR4, illustrating the importance of the conformation of the p3bv region (Figs. 3 and 4).

The crystal structure of bvPLA$_2$ indicates that the most active part of the p3bv peptide (i.e., the p3bv1 peptide) is located within a surface loop that is likely to be conformationally flexible. This surface loop is close to the interfacial recognition surface, membrane lipids (Scott et al., 1990), and also to the residues that have been involved in the binding of bvPLA$_2$ to rat brain N-type sPLA$_2$ receptors (Nicolas et al., 1997). Since the high-affinity sPLA$_2$ binding sites found in HIV-1 host cells appear related to rat brain N-type sPLA$_2$ receptors (Fenard et al., 1999), it is possible that bvPLA$_2$ first binds to its primary target through residues that are close to the p3bv1 peptide loop and then conformational changes occur, allowing the sPLA$_2$ to interact with other cellular or viral targets. In particular, this primary binding may change the conformation of the p3bv1 region, which in turn can interact with the CXCR4 receptor. Another possibility could be that the bvPLA$_2$ is internalized and degraded after its high-affinity binding to host cells and that this degradation generates bvPLA$_2$-derived peptides, such as the p3bv peptide. Interestingly, a region that is very similar to p3bv has been identified as a specific epitope for T cells after immunization with bvPLA$_2$ (Mori et al., 1993).

In conclusion, here we describe a novel peptide called p3bv that is derived from bvPLA$_2$ and inhibits T-tropic, but not M-tropic HIV-1, through its binding to CXCR4. Our results, however, suggest that the mechanisms of HIV inhibition by the p3bv peptide are probably different from the original bvPLA$_2$ complete protein. Comparisons of the antiviral properties of p3bv with those of bvPLA$_2$ suggest that the mechanism of inhibition of HIV-1 by bvPLA2 is a more complex process, which may involve several steps and various bvPLA$_2$ domains distinct from p3bv that can act separately or in concert to inhibit entry of X4 and R5 viruses into host cells. Finally, the recent identification of a novel human sPLA$_2$ that belongs to group III sPLA$_2$s and displays a significant homology with bvPLA$_2$, in particular in the p3bv peptide region (Valentin et al., 2000), suggests a role of this type of sPLA$_2$ in HIV infection. Whether this human sPLA$_2$ has an antiviral activity similar to bvPLA$_2$ will be particularly interesting to analyze in the future.

Acknowledgments

We are very grateful to Professor M. Lazdunski for a critical review of this manuscript and very fruitful discussions. We thank P. Rochet for expert technical assistance. We are also grateful to P. Charneau, N. Israel, the NIH AIDS Research and Reference Reagent Program for the generous gifts of the various plasmids and cell lines, and Y. Chvatchko for providing us with a first set of bvPLA$_2$ peptides. Special thanks are also due to Dr. Fernando Arenzana-Seisdedos for supplying the 6H8 antibody.

References

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM and Berger EA (1996) CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science (Wash DC)* **272:**1955–1958.

Berger EA, Murphy PM and Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* **17:**657–700.

Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J and Springer TA (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature (Lond)* **382:**829–833.

Chan DC and Kim PS (1998) HIV entry and its inhibition. *Cell* **93:**681–684.

Charneau P, Mirambeau G, Roux P, Paulous S, Buc H and Clavel F (1994) HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol* **241:**651–662.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, et al. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85:**1135–1148.

Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, Virelizier JL, Baggiolini M, Sykes BD and Clark-Lewis I (1997) Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* **16:**6996–7007.

Cupillard L, Koumanov K, Mattei MG, Lazdunski M and Lambeau G (1997) Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A2. *J Biol Chem* **272:**15745–15752.

Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF and Weiss RA (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature (Lond)* **312:**763–767.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di MP, Marmon S, Sutton RE, Hill CM, et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature (Lond)* **381:**661–666.

Dennis EA (1997) The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends Biochem Sci* **22:**1–2.

Doranz BJ, Grovit FK, Sharron MP, Mao SH, Goetz MB, Daar ES, Doms RW and O'Brien WA (1997) A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J Exp Med* **186:**1395–1400.

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP and Paxton WA (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature (Lond)* **381:**667–673.

Fenard D, Lambeau G, Valentin E, Lefebvre JC, Lazdunski M and Doglio A (1999) Secreted phospholipases A$_2$, a new class of HIV inhibitors that block virus entry into host cells. *J Clin Invest* **104:**611–618.

Feng Y, Broder CC, Kennedy PE and Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science (Wash DC)* **272:**872–877.

Gelb MH, Jain MK, Hanel AM and Berg OG (1995) Interfacial enzymology of glycerolipid hydrolases: lessons from secreted phospholipases A2. *Annu Rev Biochem* **64:**653–688.

Gelb MH, Min JH and Jain MK (2000) Do membrane-bound enzymes access their substrates from the membrane or aqueous phase: interfacial versus non-interfacial enzymes. *Biochim Biophys Acta* **1488:**20–27.

Howard OM, Oppenheim JJ and Wang JM (1999) Chemokines as molecular targets for therapeutic intervention. *J Clin Immunol* **19:**280–292.

Ishizaki J, Suzuki N, Higashino K, Yokota Y, Ono T, Kawamoto K, Fujii N, Arita H and Hanasaki K (1999) Cloning and characterization of novel mouse and human secretory phospholipase A$_2$s. *J Biol Chem* **274:**24973–24979.

Kini RM and Evans HJ (1989) A model to explain the pharmacological effects of snake venom phospholipases A2. *Toxicon* **27:**613–635.

Lambeau G, Barhanin J, Schweitz H, Qar J and Lazdunski M (1989) Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. *J Biol Chem* **264:**11503–11510.

Lambeau G and Lazdunski M (1999) Receptors for a growing family of secreted phospholipases A2. *Trends Pharmacol Sci* **20:**162–170.

Mori A, Thomas P, Tagaya Y, Iijima H, Grey H and Ishizaka K (1993) Epitope specificity of bee venom phospholipase A2-specific suppressor T cells which produce antigen-binding glycosylation inhibiting factor. *Int Immunol* **5:**833–842.

Murakami M, Nakatani Y, Atsumi G, Inoue K and Kudo I (1997a) Regulatory functions of phospholipase A2. *Crit Rev Immunol* **17:**225–283.

Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, Tamamura H, Yoshida N, Waki M, Matsumoto A, Yoshie O, et al. (1997b) A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med* **186:**1389–1393.

Nicolas JP, Lin Y, Lambeau G, Ghomashchi F, Lazdunski M and Gelb MH (1997) Localization of structural elements of bee venom phospholipase A2 involved in N-type receptor binding and neurotoxicity. *J Biol Chem* **272:**7173–7181.

Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier JL, Arenzana SF, Schwartz O, Heard JM, Clark LI, Legler DF, et al. (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature (Lond)* **382:**833–835.

Ross TM, Bieniasz PD and Cullen BR (1999) Role of chemokine receptors in HIV-1 infection and pathogenesis. *Adv Virus Res* **52:**233–267.

Schols D, Este JA, Henson G and De Clercq E (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Res* **35:**147–156.

Scott DL, Otwinowski Z, Gelb MH and Sigler PB (1990) Crystal structure of bee-venom phospholipase A2 in a complex with a transition-state analogue. *Science (Wash DC)* **250:**1563–1566.

Suzuki N, Ishizaki J, Yokota Y, Higashino K, Ono T, Ikeda M, Fujii N, Kawamoto K and Hanasaki K (2000) Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipase A$_2$s. *J Biol Chem* **275:**5785–5793.

Tilton B, Ho L, Oberlin E, Loestscher P, Baleux F, Clark-Lewis I and Thelen M (2000) Signal transduction by CXC chemokine receptor 4: stroma cell-derived

factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* **192:**313–324.
Tischfield JA (1997) A reassessment of the low molecular weight phospholipase A2 gene family in mammals. *J Biol Chem* **272:**17247–17250.
Valentin E, Ghomashchi F, Gelb MH, Lazdunski M and Lambeau G (1999a) On the diversity of secreted phospholipases A_2. Cloning, tissue distribution, and functional expression of two novel mouse group II enzymes. *J Biol Chem* **274:**31195–31202.
Valentin E, Ghomashchi F, Gelb MH, Lazdunski M and Lambeau G (2000) Novel human secreted phospholipase A_2 with homology to the group III bee venom enzyme. *J Biol Chem* **275:**7492–7496.
Valentin E, Koduri RS, Scimeca JC, Carle G, Gelb MH, Lazdunski M and Lambeau G (1999b) Cloning and recombinant expression of a novel mouse-secreted phospholipase A_2. *J Biol Chem* **274:**19152–19160.
Valentin E and Lambeau G (2000) Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins. *Biochim Biophys Acta* **1488:**59–70.

Address correspondence to: Dr. Alain Doglio, Laboratoire de Virologie, INSERM U526, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 2, France. E-mail: doglio@unice.fr