Journal of Peptide Science
J. Peptide Sci. **4**: 479–485 (1998)

SPC_3, an Anti-HIV Peptide Construct Derived From the Viral Envelope, Binds and Enters HIV Target Cells

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Received 12 March 1998
Accepted 4 May 1998

Abstract: SPC_3 is a peptide construct (eight branches of the GPGRAF motif) derived from the consensus sequence present at the apex of the third variable domain of the human immunodeficiency virus (HIV) envelope (Env). It presents a potent anti-HIV activity and is currently tested in phase II clinical trials (FDA protocol 257A). Its mode of action remains unclear. It was thought that SPC_3 exerts its effect both during HIV interaction with $CD4^+$ cells but also through interference either with a post-binding event or with Env processing. Accordingly, SPC_3 was supposed to be able to bind and to enter $CD4^+$ cells. In this work, we addressed these points. SPC_3 was found to interact with $CD4^+$ cell membrane with a $K_{0.5}$ value in the range of 500 nM. The binding of SPC_3 to $CD4^+$ cells involves its interaction with a cell membrane associated protein which is pronase sensitive and different from CD4. This interaction was similar from 2 to 37°C. The maximum binding occurred at acidic pH whereas the interaction was inhibited in alkaline conditions. We observed also that SPC_3 was internalized rapidly into the cells — the maximal intracell amount was reached within 30 min — where it remained stable for at least 24 h. Altogether, these data suggest that SPC_3 can exert its antiviral activity *via* interference with events occurring at the cell surface but also into the target cell. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: anti-HIV agent; Env-derived peptide; SPC_3; V_3 domain; HIV infection

INTRODUCTION

The mature human immunodeficiency virus (HIV) envelope (Env) is composed of the outer membrane glycoprotein gp120 and the transmembrane subunit gp41. gp120 mediates HIV tropism and binding to human lymphoid $CD4^+$ cells. After virus attachment to the CD4 membrane antigen, conformation changes occur within the envelope [1]. The gp41 fusion peptide can then induce the fusion between the virus and the host cell membrane, leading to HIV entry.

The third variable domain of gp120 (V_3) is a 35 amino-acid residue (aa) disulfide-bonded region which presents a conserved GPGR motif at its crown [2]. Among the various regions which are involved in Env conformation changes occurring after CD4 binding, V_3 plays a central role, partially through its interaction with the CDR3 region of CD4 [3] and chemokine receptors [4]. Based on this knowledge, monomeric linear or cyclic V_3 loop-related peptides have been synthesized to interfere with some of the cell/virus fusion steps, to alter Env functions, and hence HIV infectivity [5–8]. Among these peptides, a synthetic polymeric construction (SPC_3) containing the consensus sequence of the apex of the V_3 domain of HIV_{-1} North American/European isolates (i.e. GPGRAF) [2] inhibits potently the infection of human $CD4^+$ lymphocytes and macrophages by distantly related laboratory strains and clinical isolates of HIV_{-1} and HIV_{-2} [6–8]. This peptide is currently under phase II clinical trial (FDA protocol 257A) but its mode of action remains uncertain.

SPC_3 was shown to be a potent inhibitor of HIV infection in $CD4^+$ cell cultures even when added 1 h after initial exposure of the cells to HIV. However,

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CCC 1075–2617/98/080479-07$17.50

a more potent effect was achieved when SPC_3 is present at the onset of the infection process [6–8]. Thus, it was supposed that SPC_3 exerts its effect both during HIV interaction with $CD4^+$ cells but also through interference either with a post-binding event or with Env processing. Accordingly, SPC_3 was supposed to be able to bind and to enter $CD4^+$ cells. In this work, we addressed these points.

MATERIAL AND METHODS

Synthesis of Synthetic Polymeric Constructions

Chemical synthesis of SPC_3 (Figure 1) was performed by the solid phase technique [9] using Fmoc/*t*-butyl chemistry (reagents were from Perkin Elmer, Paris, France), as described [8,10]. The crude peptides were purified by C18 reversed-phase medium pressure liquid chromatography (Labomatic, HD–SIL 15–25 μm, 26 × 313 mm) by means of a 90 min linear gradient of acetonitrile in 0.1% (by vol.) trifluoroacetic acid from 0 to 40% at a flow rate of 6 ml/min ($\delta = 230$ nm). The homogeneity of the peptides was assessed by analytical C18 reversed-phase high pressure liquid chromatography (Merck, C18 Lichrospher 5 μm, 4 × 200 mm) by means of a 60 min linear gradient of acetonitrile in 0.1% (by vol.) trifluoroacetic acid from 0 to 60% at a flow rate of 1 ml/min ($\delta = 230$ nm). The purified peptides were characterized by both amino acid analysis after acid hydrolysis (6 M HCl/1% (mass/vol.) phenol, 20 h, 120°C), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. They were then tested in bioassays. A fully active structural analog of SPC_3, i.e. $[GPGRAF]_8$-K_4-K_2-K-Y, was labeled with 300 μCi of [^{125}I]Na in a 4 nM iodogen-coated tube for 30 min at 20°C (5 μg of peptide in 40 μl of PBS; specific radioactivity: 50 μCi/μg). Reaction was stopped with a saturating concentration of tyrosine and the peptide was gel-purified on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden).

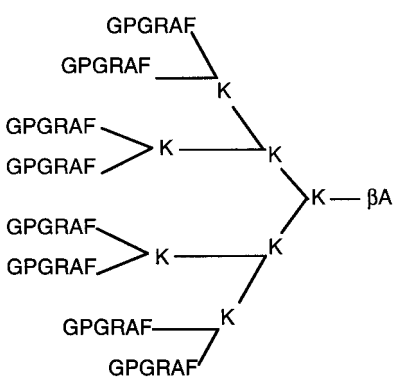

Figure 1 Synthetic Polymeric Construction 3.

Binding and Internalization of SPC_3

$CD4^+$ CEM cells (10^6/ml) were cultured in RPMI 1640 (Gibco BRL, Les Ullis, France), 5% fetal calf serum.

For binding experiments, cells (2×10^6/200 μl) were incubated at 22°C with [^{125}I]SPC_3 in cell culture medium supplemented with 0.1% NaN_3 (a metabolic poison that blocks energy metabolism, and consequently endocytosis [11–14]), in the absence or in the presence of various concentrations of unlabeled SPC_3 (10^{-4}–10^{-8} M). Cells were then washed twice with PBS 0.1% NaN_3 and bound radioactivity was counted. All conditions were performed in duplicate. Binding experiments were also performed in melting ice or at 37°C. Alternatively, (i) to assess whether the molecule which interacts with SPC_3 is a protein, cells were treated by pronase (Sigma, St Louis, MO; 400 μg/200 μl final volume) for 15 min at 37°C before washing [15]. Cells were then incubated with [^{125}I]SPC_3 in melting ice and processed as described above; (ii) to study the interaction of SPC_3 with sCD4 (this 55 kDa glycoprotein corresponds to the recombinant soluble form of the human CD4 membrane antigen; it comprises the two N terminal domains involved in HIV binding but lacks the transmembrane region; sCD4 is a gift from Ian Jones, NERC, Oxford, UK): [^{125}I]SPC_3 (5 ng) was pre-incubated with sCD4 (50 ng to 5 μg/100 μl PBS) for 1 h at 37°C before co-incubation with $CD4^+$ cells for 1 h. Cells were then processed as described above; (iii) to study the effect of pH conditions on the binding of [^{125}I]SPC_3, cells were incubated with the peptide for 1 h in cell culture medium supplemented with NaN_3 0.1% and adjusted at various pH (4.6–9.1). Cells were then processed as described above.

For internalization experiments, cells were incubated as described above. Cell associated radioactivity when incubation was performed in the presence of the metabolic poison NaN_3 — a condition that impairs endocytosis — was considered as membrane-associated SPC_3 (see validation of the methodology below). [^{125}I]SPC_3 present within the cell was estimated at various times after addition of [^{125}I]SPC_3 in the cell culture as follows: 'cell-associ-

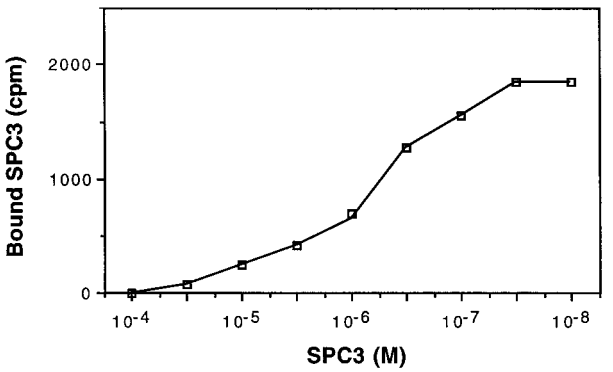

Figure 2 Evaluation of the $K_{0.5}$ value of the SPC_3 binding to CD4$^+$ cell membrane. CD4$^+$ CEM cells were incubated for 1 h with [^{125}I]SPC_3 and various amounts of unlabeled SPC_3 (10^{-4}–10^{-8} M) in the presence of 0.1% NaN_3. Cell bound radioactivity was then counted (the data presented are the mean of $n = 3$ experiments; duplicates were performed; background: 350 ± 100 cpm).

ated radioactivity obtained when incubation was performed in the absence of NaN_3 "minus" cell-associated radioactivity obtained when incubation was performed in the presence of NaN_3'. Alternatively, cells were incubated with [^{125}I]SPC_3 for 2 h, washed and then incubated in cell culture medium for 24 h. Cells were then analysed with 0.1% SDS at 2°C. The lysate was analysed by Sephadex G-25 M chromatography (PD10 column, Pharmacia; elution was performed in PBS, fraction: 1 ml). The supernatant of cells incubated for 24 h with [^{125}I]SPC_3 was similarly analysed. As a control, [^{125}I]SPC_3 was incubated for 24 h in cell culture medium and then analysed.

RESULTS

SPC_3 Binding to the CD4$^+$ Cell Surface

SPC_3 was tested for its ability to bind CD4$^+$ CEM cell membrane in the presence of NaN_3 (0.1%), a condition that impairs endocytosis and thus excludes its influence on the amount of cell-associated [^{125}I]SPC_3. A saturation curve was obtained by incubating cells with [^{125}I]SPC_3 and varying the concentration of unlabeled SPC_3 (10^{-4}–10^{-8} M): SPC_3 bound cell membrane in a dose dependent manner with a $K_{0.5}$ value in the range of 500 nM (Figure 2).

Under these conditions (NaN_3 0.1%), when CEM cells were incubated with [^{125}I]SPC_3, the maximum binding was reached within 30 min (Figure 3). The ability of SPC_3 to bind cells was independent on the temperature and occurred similarly at +2°C (Figure 3), 22°C (data not shown) and 37°C (Figure 3).

To determine the nature of the cell membrane-associated compound interacting with SPC_3, cells were first treated by pronase to cleave outer-membrane proteins before incubation with [^{125}I]SPC_3 (Figure 4A). Pronase treatment abolished the subsequent binding of SPC_3. This indicates that SPC_3 binds a protein at the cell surface. As a control, SPC_3 at 300 nM concentration diminished by about 50% [^{125}I]SPC_3 binding, in agreement with the data presented above (see Figure 2).

Validation of the methodology used is shown (Figure 4B): the sum of radioactivity bound to cells in the presence of NaN_3 ('membrane-bound SPC_3') and of the radioactivity associated to living cells treated by pronase ('internalized SPC_3') was equal to the radioactivity associated to cells in the control conditions ('membrane bound SPC_3' plus 'internalized SPC_3'). This is consistent with the fact that 0.1% NaN_3 impaired SPC_3 entry into the cell and that pronase treatment was efficient.

Finally, pre-incubation of SPC_3 with sCD4 for 1 h did not alter its subsequent ability to bind CD4$^+$ cells (data not shown), indicating that CD4$^+$ is not the target receptor of SPC_3.

To further characterize the structure which binds SPC_3, we studied the effect of pH conditions on SPC_3 binding. Cells were incubated with [^{125}I]SPC_3 in various pH conditions. SPC_3 binding was maximal for acidic pH (below pH 5.5) (Figure 5). A plateau corresponding to a 50% maximum binding

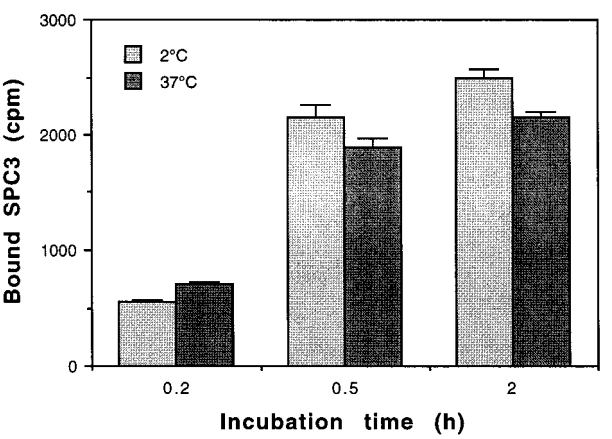

Figure 3 Effect of temperature on SPC_3 binding. CD4$^+$ CEM cells were incubated for 30 min, 1 or 2 h with [^{125}I]SPC_3 in the presence of 0.1% NaN_3 at 2 or 37°C. Cell bound radioactivity was then counted ($n = 3$).

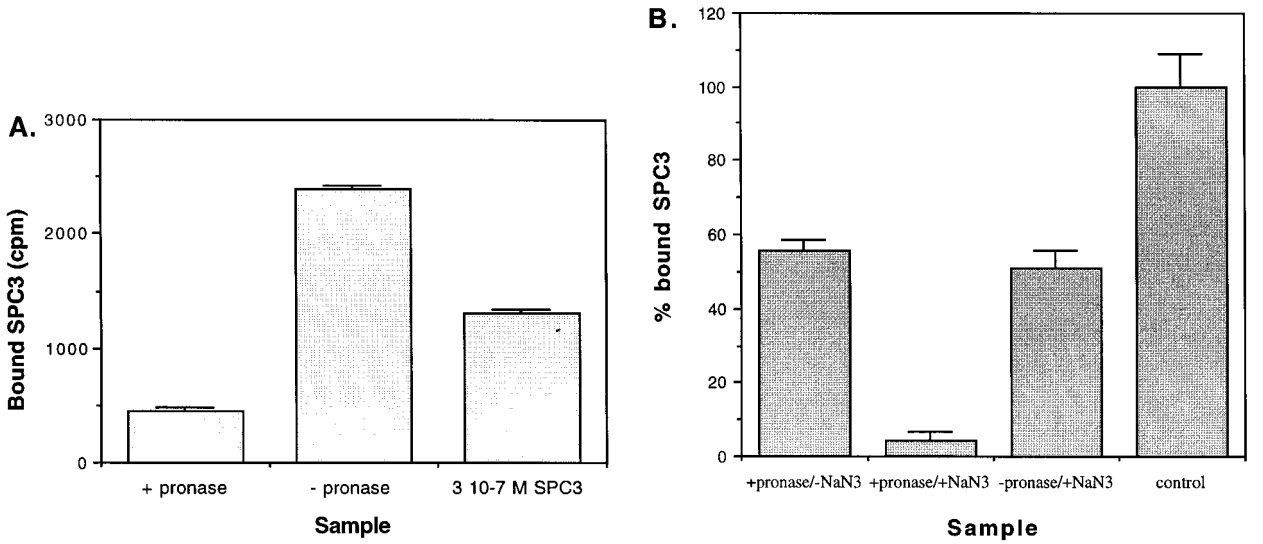

Figure 4 Effect of pronase treatment of $CD4^+$ CEM cells onto their subsequent ability to bind SPC_3. (A) Cells were treated by pronase (+pronase), or not (−pronase), under conditions which cleave outer-membrane proteins. After washing, cells were incubated with $[^{125}I]SPC_3$ for 1 h in melting ice (no internalization). As a control, a 3×10^{-7} M concentration of SPC_3 was added during incubation of $[^{125}I]SPC_3$ with untreated cells. Bound radioactivity was counted ($n = 3$). (B) Cells were incubated for 1 h with $[^{125}I]SPC_3$ in the presence of NaN_3 (+NaN_3; no internalization) or not (−NaN_3). After a wash, cells were treated by pronase (+pronase) or not (−pronase). The control situation corresponded to cells incubated with $[^{125}I]SPC_3$ in the absence of NaN_3 and without pronase treatment. Cell bound radioactivity was then counted ($n = 2$).

was obtained for pH values ranging from 6 to 7. Alkaline pH values abolished $[^{125}I]SPC_3$ binding. These data suggest that (i) the binding of SPC_3 to its receptor is sensitive to pH conditions presumably by pH-induced conformation changes of the SPC_3 binding sites or (ii) SPC_3 binds a protein non covalently associated at the membrane of CEM cells (such proteins display increased cell association at low pH values whereas high pH values induce their dissociation). To address this point, cells were incubated first at either alkaline, neutral or acidic pH for 1 h and then washed before addition of $[^{125}I]SPC_3$ at pH 7.2 for 1 h. As shown in Figure 5, these conditions did not influence the subsequent binding of SPC_3 to cells at pH 7.2. Similarly, pre-incubation of cells at alkaline pH to remove non covalently cell associated proteins did not modify the subsequent characteristics of SPC_3 binding at various pH. Thus, the ability of SPC_3 to bind cells is influenced by pH conditions.

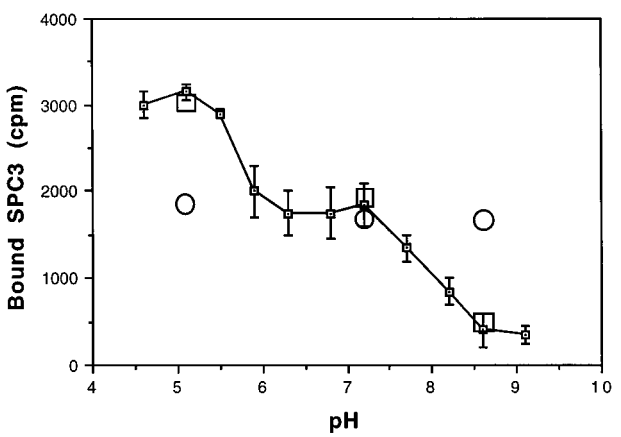

Figure 5 Effect of pH on SPC_3 binding. $CD4^+$ CEM cells were incubated for 1 h with $[^{125}I]SPC_3$ in various pH conditions in the presence of 0.1% NaN_3. Alternatively, (i) cells were incubated for 1 h at pH 5.5, 7.2 or 9 before incubation with SPC_3 for an additional 1 h period at pH 7.2 (○) or (ii) cells were incubated for 1 h at pH 9 before incubation with SPC_3 for an additional 1 h period at various pH (□). Cell bound radioactivity was then counted ($n = 4$).

Internalization of SPC_3 into $CD4^+$ cells

Cells were incubated with SPC_3 in the presence of NaN_3 (to determine the amount of membrane-bound SPC_3), or not, in cell culture medium. According to Figure 4B, the amount of SPC_3 which entered cells was determined by subtraction, from the signal corresponding to radioactivity associated to living cells, of the signal obtained in the presence of NaN_3. As shown Figure 6, this process was rapid,

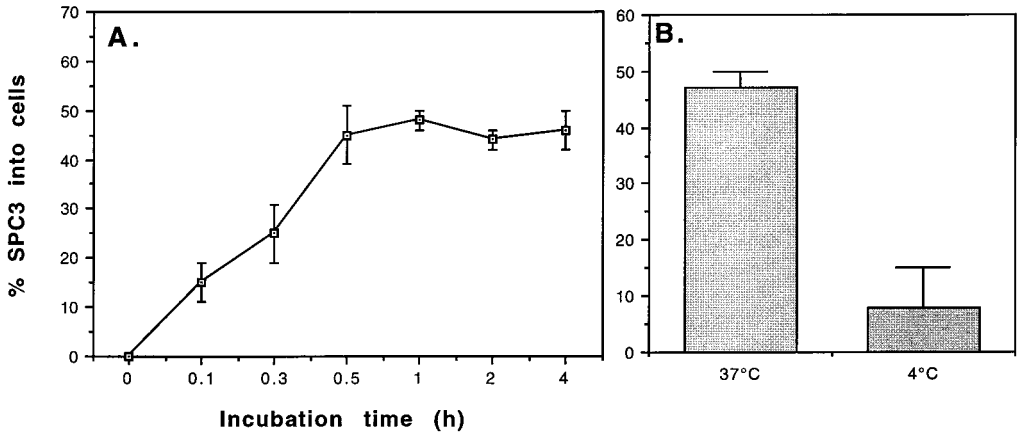

Figure 6 Internalization of SPC_3 into CD4$^+$ CEM cells. (A) The amount of internalized [^{125}I]SPC_3 was indicated as a percentage relative to the total cell-associated SPC_3 radioactivity. It was calculated by subtraction, from the signal corresponding to radioactivity associated with living cells, of the signal obtained in the presence of 0.1% NaN_3 (membrane bound SPC_3) ($n = 7$). (B) [^{125}I]SPC_3 internalization after a 2 h incubation at $+4$°C and $+37$°C.

with a plateau reached within 30 min. As a control, incubation of cells with [^{125}I]SPC_3 at $+4$°C inhibited internalization of the peptide compound.

The radioactivity remaining associated to the cells 24 h after a pulse incubation for 2 h with [^{125}I]SPC_3 (10^5 cpm) was studied. In a representative experiment, about 4000 cpm were associated with cells at the end of the pulse incubation with [^{125}I]SPC_3 and about 3000 cpm remained associated 24 h later. The corresponding cell lysate was analysed by chromatography (Figure 7). The elution profile indicates that [^{125}I]SPC_3 was rather stable under these conditions. A similar profile was obtained when [^{125}I]SPC_3 was incubated in culture medium in the absence of cells. Of note, incubation of SPC_3 for 3 days at 37°C in cell culture medium in the absence of cells did not have any effect on its subsequent anti-HIV activity (data not shown). In contrast, the profile corresponding to [^{125}I]SPC_3 incubated for 24 h in cell culture medium in the presence of cells was different.

Despite the postulate that SPC_3 exerts its activity through an enhanced ligand avidity, the ability of SPC_3 to bind a component present at the membrane of target cells has never been investigated in details. It has been reported in [7] that a fluorescent derivative of SPC_3 was able to interact with CD4. However, this finding is controversial since such chemical modification interferes with SPC_3 activity.

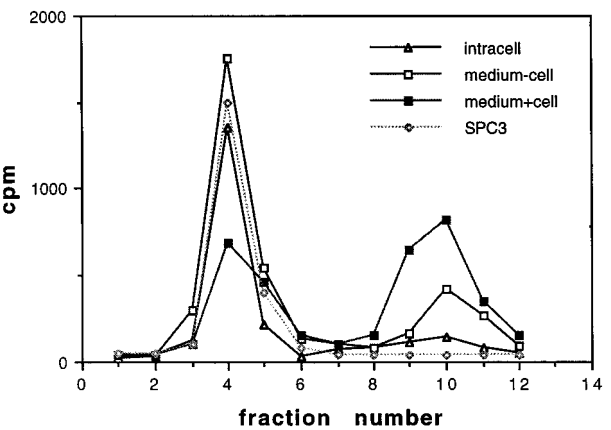

Figure 7 Chromatography profiles of [^{125}I]SPC_3 present in the cells or in their supernatant after a 24 h incubation. [^{125}I]SPC_3 was incubated with CD4$^+$ CEM cells for 2 h; cells were then washed and cell-associated radioactivity (intracell) was analysed 24 h later by G25 chromatography after cell lysis with 0.1% SDS at 2°C. Alternatively, [^{125}I]SPC_3 incubated in cell culture medium in the absence (medium – cell) or in the presence (medium + cell) of cells for 24 h was similarly studied. As a control, [^{125}I]SPC_3 (SPC_3) was analysed. A representative experiment ($n = 2$) of each condition is presented.

DISCUSSION

Originally, the rationale for using V_3 SPCs instead of monomeric V_3 peptides as anti-HIV agents was that they may have enhanced ligand avidity due to the high local density of binding sites. A similar assumption was validated in [16]; multimerization of a short peptide derived from the laminin B1 chain resulted in a large increase of its anti-tumor properties.

Because SPC_3 inhibits various HIV isolates with sequences other than GPGRAF at the crown of their V_3 domain [6–8], it is reasonable to hypothesize that such a potent anti-HIV activity was due to the ability of SPC_3 to interact with a cellular component of human lymphoid $CD4^+$ cells (but not to viral determinants), thus by-passing the problems inherent in envelope variability. Our study supports this hypothesis; we observed that SPC_3 was able to bind specifically a membrane proteic component present on $CD4^+$ human lymphoid cells with a $K_{0.5}$ value of 500 nM. This $K_{0.5}$ value is similar to those obtained with various short bioactive peptides interacting with their receptors, e.g. KDEL-derived peptides and their receptors [17] which are involved in the cell sorting of various cell resident proteins. While this binding was not temperature dependent, it varied according to pH values and was optimal for acidic pH conditions. Such binding properties are similar to those described for the interaction of KDEL peptides with the erd2 receptor [18]. Since the overall charge of SPC_3 is positive, it may interact with the acidic CDR3 region of CD4, as previously suggested [7]. However, despite the previous hypothesis that the V_3 region interacts with this domain [3,19], we failed to detect any interaction between CD4 and SPC_3 in competition experiments. These data agree with the fact that SPC_3 binds $CD4^-$ hamster cell lines (unpublished data), and with the results reported in Yahi *et al.* [8] and Barbouche *et al.* [13].

Internalization occurred and rapidly reached a plateau. This observation is in line with the hypothesis that SPC_3 interferes also with the HIV infection process at an intracellular level [6–8] different from Env folding and membrane expression [13]. After internalization, SPC_3 appeared to be stable within the cells.

It remains possible that SPC_3 interferes with V_3-related conformation changes, including V_3 cleavage. SPC_3 may inhibit also either Env interaction with a possible coreceptor during post-internalization events [20], or Env synthesis as reported for other Env-derived peptide constructs [14,21]. These points are under investigation.

Acknowledgements

We thank Dr M.-J. Papandréou and M.H. Mezghrani for discussions during this study. This work was supported by the ANRS (grants to EF and RM). RB is an associate investigator of the CNRS (CIES).

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