Pseudopeptide TASP Inhibitors of HIV Entry Bind Specifically to a 95-kDa Cell Surface Protein*

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The template assembled synthetic peptide constructs (TASP), pentavalently presenting the tripeptide KPR or RPK, are potent and specific inhibitors of human immunodeficiency virus (HIV) infection by preventing viral entry into permissive cells. Here the 5[KW(CH2N)PR]-TASP construct, Ψ(CH2N) for reduced peptide bond, was used in studies to demonstrate its specific binding to a 95-kDa cell surface protein ligand. Compared to its non-reduced 5[KPR]-TASP counterpart, the pseudopeptide 5[KW(CH2N)PR]-TASP manifested higher affinity to bind to its cell surface ligand, increased activity to inhibit HIV infection, and resistance to degradation when incubated in serum from an HIV-1 seropositive individual. In ligand blotting experiments, the biotin-labeled 5[KW(CH2N)PR]-TASP identified a single 95-kDa protein in crude cell extracts. This 95-kDa protein (p95) is expressed on the cell surface since surface iodination of cells resulted in its labeling, and moreover, following incubation of cells with the biotin-labeled 5[KW(CH2N)PR]-TASP, the p95 TASP complex was recovered by affinity chromatography using avidin-agarose. All anti-HIV TASP constructs but not their control derivatives affected the binding of biotin-labeled 5[KW(CH2N)PR]-TASP to p95, thus emphasizing the specific nature of this binding. Since 5[KΨ(CH2N)PR]-TASP does not interact with HIV-envelope glycoproteins, our results suggest that TASP inhibitors mediate directly or indirectly a block in HIV-mediated membrane fusion process by binding to the cell surface expressed p95.

HIV is an enveloped virus that infects target cells by the fusion of viral and cellular membranes. This fusion is initiated by the binding of HIV external and transmembrane envelope glycoprotein complex to the CD4 receptor and also is dependent on the presence of species-specific cofactors on the cell surface (for a review, see Ref. 1). The external envelope glycoprotein contains the binding site for the CD4 receptor and a hypervariable region of about 36 amino acids referred to as the V3 loop (2). The transmembrane glycoprotein contains a potential fusion peptide at its amino terminus, which is implicated in the membrane fusion process (3). The external and transmembrane glycoproteins (gp120/gp41 for HIV-1) are associated in a noncovalent manner to generate a functional complex. The gp120-gp41 complex is essential for binding of HIV particles to the cell membrane, whereas in HIV-infected cells, this complex expressed on the cell surface is responsible for the initiation of cell to cell membrane fusion, leading to the formation of syncytia. The cell surface-expressed gp120-gp41 complex is also responsible for the initiation of cell death by apoptosis in HIV-infected cell cultures (4). The V3 loop plays a critical role in these well-defined functions of the gp120-gp41 complex (1, 2, 5), and it has been proposed that it might be implicated in post-CD4 binding events by interacting with specific cell surface proteins (2).

The V3 loop of different HIV-1 and HIV-2 isolates contains a highly conserved RP dipeptide motif at its NH2-terminal end (6). In view of this, we have designed and synthesized a construction referred to as template assembled synthetic peptide (TASP) in which templates such as KKKGPKEKGC and KKKKGC were employed to covalently anchor arrays of tripeptides (RPR, RPK, or KPR) at the e-amino group of the lysine residues in the templates (7). The pentavalent presentation, 5(RPR)-, 5(RPK)-, or 5(KPR)-TASP molecules, manifested maximum inhibitory activity on infection of cells by HIV-1 and -2 but not by simian immunodeficiency virus isolates, thus emphasizing the specific nature of these TASP inhibitors. In already infected cells, the TASP inhibitors also blocked syncytium formation and the occurrence of apoptosis (7). These observations indicated that these TASP inhibitors block a defined target that should be implicated in the functioning of the gp120-gp41 complex to initiate viral entry, syncytium formation, and apoptosis. Structure and inhibitory activity relationship studies using analogs of 5(KPR)-TASP indicated that the two basic residues Lys and Arg in the tripeptide are essential and can be replaced by each other and that their positively charged side chains play a critical role in the inhibitory structure. Interestingly, replacement of Leu amino acid residues by Asp amino acids or the reduction of the peptide bond between the first two amino acids of the tripeptide generated pseudopeptide-TASP analogs active at submicromolar concentrations on HIV infection (7). By the use of FITC- and biotin-
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labeled [5K(V(CH3)N)PR]-TASP constructs, we demonstrate here that the TASP inhibitors bind specifically to a 95-kDa, cell surface protein (p95). The identity of p95 remains to be elucidated. However, because the TASP inhibitors block both HIV envelope-mediated virus to cell and cell to cell membrane fusion processes and the initiation of apoptosis (7), it can be suggested that p95 is implicated as a potential cofactor of CD4 in the functioning of the gp120-gp41 complex to initiate membrane fusion and thus viral entry. Consistent with this, [5K(V(CH3)N)PR]-TASP binds the binding sites of HIV particles to CD4+ permissive cells at the same efficiency as an anti-CD4 monoclonal antibody.

MATERIALS AND METHODS

Cells and HIV Infection—CEM cells (clone 13) derived from human lymphoid cell line CEM and MOLT4-T4 clone 8 cells selected for high level of CD4 expression (both cell lines were provided by L. Montagnier, Institut Pasteur, France) were cultured in suspension RPMI 1640 medium (BioWhittaker, Verviers, Belgium). Human HeLa cells were grown in monolayer cultures in Dulbecco’s medium. Human peripheral blood mononuclear cells (PBMC) from a healthy donor were stimulated by phytohemagglutinin (PHA) and cultured in RPMI 1640 medium containing 10% (v/v) T cell growth factor (Biozol) (7). All cells were cultured with 10% (v/v) heat-inactivated (95°C for 30 min) fetal calf serum.

Infection of CEM cells with the HIV-1 Lai isolate was carried out as described previously (7). For the assay of the inhibitory effect of the TASP constructs, CEM cells were first incubated (15 min, at room temperature) in the presence of different concentrations of each construct before infection using one synchronous dose of HIV-1 Lai as described before (8). HIV production was estimated at 4 days post-infection (p.i.) by monitoring the HIV-1 major core protein p24 in the culture supernatant (7). The concentration of p24 was measured by p24 Core Profile ELISA (Du Pont).

Buffers—Buffer E contained 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, aprotinin (1000 units/ml), and 0.5% Triton X-100. Triton X-bufferted saline contained 25 mM Tris-HCl, pH 7.0, 137 mM NaCl, and 3 mM KCl. Fluorescence-activated cell sorter (FACS) buffer contained 1% bovine serum albumin and 0.01% sodium azide in phosphate-buffered saline (PBS). Two-fold concentrated electrophoresis sample buffer contained 125 mM Tris-HCl, pH 6.8, 2 M urea, 1% SDS, 0.1% bromphenol blue, 0.1 mM β-mercaptoethanol, and 20% glycerol (v/v). Buffer GF contained 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, and 0.1% Triton X-100.

Preparation of Cell Extracts—Cells were first washed extensively in PBS before lysis in buffer E (100 μL x 10⁶ cells), and the nuclei were pelleted by centrifugation (1,000 × g for 5 min). The nuclei-free supernatant was then further centrifuged at 12,000 × g for 10 min, and the supernatant was stored at −80°C. Routinely, cell extracts were prepared 24 h after cell passage.

Cell Surface Iodination of Cells—CEM cells (10⁶ cells) were washed with PBS (2 × 25 ml), and the pellet was suspended in 20 ml of PBS containing 10 mM p-glucose and 2 μCi of ¹²⁵I (100 μCi/ml; Amersham), 2 units of lactoperoxidase, and 2 units of glucose oxidase (Calbiochem-Behring). After 10 min of incubation at 22°C, cells were washed in PBS, and extracts were prepared as above.

Synthesis of TASP Constructs—Synthesis of the different TASP constructs and the measurement of their inhibitory activity on HIV infection were as described previously (7). The following TASP constructs were included in this report: 1) control peptides which manifest no little activity against HIV infection, such as [5Q(π)-Q]-, [5KQ]-, and [5K(π)]-TASP; and 2) peptides which are potent inhibitors of HIV entry and infection, such as [5K(π)]- and [5K(V(CH3)N)PR]-TASP. For the preparation of the biotin-labeled constructs, biotin was incorporated at the beginning of the synthesis by coupling the Fmoc (N-(9-fluorenyl)-methoxycarbonyl)-L-lys(Biotin)-OH derivative (Neosystem, Strasbourg, France) on the resin prior to the assembly of the template. Thus the biotinylated TASP constructs were labeled at the COOH terminus of the template.

Ligand Blotting—Crude cell extracts were diluted in 2-fold concentrated electrophoresis sample buffer and analyzed by polyacrylamide gel electrophoresis (PAGE) in SDS to be electrophoretically transferred to 0.22 μm polyvinylidene difluoride sheets (Bio-Rad). The electrophoretic blots were saturated with casein-based blocking buffer (GENSYS) overnight at 4°C. In order to further saturate nonspecific binding sites, the blots were first incubated at room temperature in blocking buffer containing 5 μM 5K(π)-TASP. After 2 h, the biotin-labeled [5K(V(CH3)N)PR]-TASP (5 μM) was added to this solution, and the blots were incubated for another 2 h at 4°C. The sheets were subsequently washed 3 times (10 min each) in Tris-buffered saline containing 0.05% (v/v) Tween 20, followed by 2 washes (10 min each) in Tris-buffered saline before washing biotin-labeled horse-radish peroxidase complex and light-based enhanced chemiluminescence reagents as provided by the manufacturer (Amersham). The enhanced light signal produced was then captured on the autoradiography film (Hyperfilm-β-MP from Amersham Corp.).

Isolation of the Cell Surface Ligand of TASP—Twenty-four hours after seeding, CEM cells were washed extensively with PBS before incubation (50 x 10⁶ cells/300 μl of FACS buffer) at 4°C for 30 min with different concentrations of the biotin-labeled TASP molecule. Cells were then washed in FACS buffer (2 x 15 ml), and nuclear-free cell extracts were prepared using buffer E (150 μl). Such extracts were first diluted in PBS (600 μl) before the addition of 100 μl of avidin-agarose (ImmunoPure immobilized avidin from Pierce) to capture the biotin-labeled TASP complexed to its cell surface ligand. These suspensions were incubated at 4°C for 2 h, and the avidin-agarose bound proteins were washed batchwise with PBS (5 x 5 ml). Finally, the avidin-agarose pellet was resuspended in 100 μl of 2-fold concentrated electrophoresis sample buffer and heated at 95°C for 5 min. The eluted proteins were analyzed by SDS-PAGE, and the TASP ligand was revealed by ligand blotting.

FITC Labeling of TASP Constructs—[5K(V(CH3)N)PR]-TASP was labeled with fluorescein isothiocyanate (FITC, Sigma) by incubating stoichiometric concentrations (2.5 mM) of each product in 50 mM NaHCO₃, pH 9.5, at 20°C for 2 h (in the dark). This solution (400 μl) was then transferred to a Microcon Model 3 filter sieve (Amicon, Inc., MA) with a molecular mass cut-off of 3,000 Da and centrifuged at 12,000 × g for 30 min to filter unbound FITC. The concentrated material was diluted 20-fold in distilled water and purified again using the Microcon filter.

Detection of Cell Surface Antigens—Phycocerythrin (PE)-labeled mAb Ta1 (IgG1; from Coulter, Miami, FL) was used to detect CD26 (9). Two different FITC-labeled mAbs specific to the CD4 receptor were used, mAbs OKT4 and OKT4A (both IgG1; Ortho Diagnostics Systems, Raritan, NJ). In all experiments, PE-labeled mAb B4 (IgG1), specific for CD19 (Couler), was used as a control for PE-labeled mAb Ta1, and FITC-labeled mouse isotype control antibody MCG1 (IgG1; Immuno Quality Products) was used as a control for FITC-labeled mAbs OKT4 and OKT4A. Cells were incubated with FITC- or PE-labeled mAbs in the FACS buffer at 4°C for 30 min. The cells were then washed twice with PBS and fixed in 1% formaldehyde in PBS and applied to an FACS scanflowcytometer (Beckton Dickinson, Mountain View, CA). For each sample, 10,000 cells were analyzed with Lysis II Software (Becton Dickinson).

In order to assay for the binding of FITC- or biotin-labeled TASP inhibitors to a cell surface antigen, different cells were washed in PBS, suspended in FITC-labeled N 5 x 10⁶ cells/100 μl containing 0.5 μM FITC-labeled or different concentrations of the biotin-labeled TASP constructs and incubated at 4°C for 30 min. The cells were then washed twice with FITC buffer and fixed in 1% formaldehyde in FITC buffer. The FITC-labeled TASP constructs were analyzed as above, whereas the biotin-labeled TASP constructs were revealed by using streptavidin–FITC complex (Amersham). The fluorescence intensity was monitored by FACS analysis using Lysis II Software (see Figs. 3 and 4 and Tables 1 and 2) or Cell Quest™ Software (Becton Dickinson and Macintosh Computer) (see Fig. 5).

Protease Treatment of Cells—Protease treatment of CEM and MOLT cells was essentially as described previously (10) with slight modifications. Briefly, cells were washed in PBS and in RPMI 1640 medium containing 1 mM EDTA before treatment with trypsin (Sigma; 2.5 mg/ml at 20°C for 5 min), proteinase K (Boehringer Mannheim GmbH, Germany; 0.2 mg/ml at 37°C for 30 min), or Pronase E (Sigma, 0.1 mg/ml at 37°C for 45 min). The reactions were stopped by 10-fold dilutions in RPMI 1640 containing 10% fetal calf serum. Cells were then washed in PBS and FACS buffer and processed for FACS analysis.

Gel Filtration Chromatography—A Superose 6 column (1.6 x 50 cm) from Pharmacia Biotech Inc. was equilibrated with buffer GF as described before for the bed volume (V₀) of 100 ml. The gel was calibrated using extracts (prepared in Buffer E) supplemented with the molecular mass markers catalase, 202 kDa, and bovine serum albumin, 68 kDa. Elution was performed in buffer GF by collecting 1 ml fractions/2 min, with the void volume (Vₐ) and total column elution volume (Vₐ) at 36 and 114 ml, respectively. Aliquots from each fraction were analyzed by ligand blotting using biotin-labeled [5K(V(CH3)N)PR]-TASP. Aliquots were also assayed for dipetidyl peptidase IV (DPP IV) activity of CD26.
and DPP IV-β by the cleavage of Gly-Pro-para-nitroanilide as described previously (11). Under these experimental conditions, CD26 and DPP IV-β eluted as monomers of 110 and 82 kDa, respectively, and these were used as convenient markers to monitor the elution profile of the TASP ligand p95.

**Plasma Membrane Preparation**—CEM cells (300 x 10^6) were washed in PBS before homogenization to prepare plasma membranes, as described before (11). The presence of TASP ligand p95 was revealed by ligand blotting using aliquots corresponding to material from 10^6 cells.

**Preparation of 125I-Labeled gp120—Recombinant gp120 (Neosystem)** was radiolabeled with the Bolton-Hunter reagent (DuPont NEN) according to the technique described by the manufacturer. To study the binding of gp120 to the CD4 receptor, CEM cells (5 x 10^5), which express high levels of CD4, were incubated in the culture medium with 125I-labeled gp120 (50 ng, 10 Ci/mg) at 37 °C for 1 h. Cells were then washed twice in PBS (5 ml), and cytoplasmic extracts were prepared by disruption of cell pellets in buffer E (125 μl). Aliquots (25 μl, corresponding to material from 10^6 cells) were diluted 10-fold in concentrated electrophoresis buffer and were analyzed by SDS-PAGE. The binding of 125I-labeled gp120 to the CD4 receptor was then revealed by autoradiography (12). The 125I-labeled gp120 band was also quantitated in a PhosphorImager (Molecular Dynamics).

**Binding of HIV Particles to CEM Cells**—CEM cells (5 x 10^5) in culture medium (1 ml) were preincubated (at 37 °C for 15 min) in the absence or presence of 5 x 10^-6 M 5(CH2N)PR]-TASP, and gp120 (100 ng/ml) was added followed by addition of HIV-1 Lai (corresponding to 25 ng of p24). After incubation at 37 °C for 1 h with gentle shaking, cells were diluted 10-fold in the culture medium and pelleted by centrifugation. Cells at 4 °C were washed once in RPMI 1640 medium (5 ml) containing 1 mM EDTA and then washed twice in RPMI 1640 medium (2 x 5 ml). Cell extracts were prepared in buffer E (50 μl), the nuclei were pelleted by centrifugation, and the supernatant was assayed for the concentration of p24. It should be noted that under these experimental conditions, the values for the bound virus represent particles bound on the cell surface as well as particles (or cores) entered into cells.

**RESULTS AND DISCUSSION**

**5K(CH2N)PR-TASP Blocks HIV Entry by Inhibiting the Membrane Fusion Process**—We have recently reported that 5K(CH2N)PR]-TASP and related TASP-inhibitors block HIV entry and thus infection (7). Such inhibition of viral entry could be demonstrated by different experimental approaches. For example, HIV entry monitored by the intracellular concentration of p24 (HIV-1 major core protein), following 1 h incubation of CEM cells with the virus, is inhibited almost completely in the presence of 5–10 μM 5K(CH2N)PR]-TASP. Similarly, viral entry monitored by the β-galactosidase activity in HeLa/CD4+ cells expressing the bacterial lacZ gene placed under the control of the HIV-1 LTR is inhibited by at least 90% at similar concentrations of 5K(CH2N)PR]-TASP (data not shown; as described in Ref. 7). Otherwise, addition of 5K(CH2N)PR]-TASP after the viral entry process does not affect virus infection, monitored by the production of virus at 4 days post-infection (as shown in Fig. 2 in Ref. 7). Here, we further investigated the timing of the inhibitory effect of 5K(CH2N)PR]-TASP during the HIV entry process. Addition of 5 μM 5K(CH2N)PR]-TASP to cells 1 h before or together with the virus resulted in more than 90% inhibition of virus production. On the other hand, addition of 5K(CH2N)PR]-TASP at 2, and 4 h post-infection reduced its inhibitory effect, and when added at 8 h, there was almost no effect (data not shown; Ref. 7). The period of 8 h is necessary for HIV-1 entry in at least 90% of target cells in the CEM cell culture (8). The effect of 5K(CH2N)PR]-TASP on the viral entry process is most probably the consequence of the inhibition of the membrane fusion process. In accord with this, we have previously provided evidence to demonstrate that 5K(CH2N)PR]-TASP efficiently blocks the gp120-gp41-mediated membrane fusion observed in cocultures of chronically HIV-1-infected cells with uninfected CD4+ cells (see Fig. 6 in Ref. 7).

In order to further investigate the mechanism of inhibition of HIV entry, we studied the effect of 5K(CH2N)PR]-TASP on the binding of gp120 and HIV-1 particles to CD4+ CEM cells. In the first set of experiments, cells were incubated with different concentrations of 5K(CH2N)PR]-TASP before further incubation with 125I-labeled gp120. Under these experimental conditions, the binding of 125I-labeled gp120 was specific since it was inhibited by anti-CD4 mAb OKT4A, which is known to block the binding of gp120 at the CD4 receptor (12, 13). The binding of gp120 was not affected at 20 μM 5K(CH2N)PR]-TASP, whereas there was a slight inhibition of binding at higher concentrations (Fig. 1A). To investigate the binding of virus to cells, CEM cells were incubated with HIV-1 particles for 1 h. Cells were then washed extensively, and the bound virus (including that which was entered into cells) was estimated by the concentration of p24 in cell lysates. As in the binding of gp120, the binding of virus was specific since it was inhibited (75%) by mAb OKT4A. Interestingly, at 10 μM 5K(CH2N)PR]-TASP, the binding of HIV particles was also inhibited (78%) at a similar extent as that exerted by mAb OKT4A alone or when 5K(CH2N)PR]-TASP was used combined with mAb OKT4A (Fig. 1B). Thus, the 22% residual binding in the presence of 5K(CH2N)PR]-TASP should represent unspecific binding. It is plausible, therefore, to consider that 5K(CH2N)PR]-TASP inhibits the gp120-gp41-mediated membrane fusion by affecting the interaction of this complex with CD4+ cells.

The discrepancy between the binding results of soluble gp120 and viral particles to CEM cells (Fig. 1) indicates that gp120 complexed to gp41 on the surface of viral particles does not have the same conformational restrictions as the soluble gp120. Thus, experiments using soluble gp120 should be interpreted cautiously.

**Specific Binding of 5K(CH2N)PR]-TASP to a Cell Surface Protein**—By FACS analysis, we show that the FITC-labeled...
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FIG. 2. Specific binding of 5[Kψ(CH2N)PR]-TASP to the cell surface. The FITC-labeled 5[Kψ(CH2N)PR]-TASP (referred to here as P19*) at 0.5 μM was added to cultures of different cell lines, CEM (sections 1, 2, 3, and 4), MOLT4 (section 2), and HeLa (section 5), or on the third day of PHA-stimulated PBMC (section 6) in the absence or presence of 50 μM unlabeled constructs as indicated: 5[Kψ(CH2N)PR]-TASP, P19, 5[KGQ]-TASP, P18, and 5[KPR]-TASP, P1. The fluorescence intensity was monitored by FACS analysis. Peak C gives the autofluorescence of each cell type incubated with unlabeled 0.5 μM 5[Kψ(CH2N)PR]-TASP. The ordinates give the relative cell number, whereas the abscissa give the relative fluorescence intensity. Note that all the different anti-HIV TASP constructs that manifested inhibitory activity on HIV infection (described in Ref. 7) could prevent the binding of FITC-labeled 5[Kψ(CH2N)PR]-TASP to cells when added in excess (at 50-100 μM concentrations).

5[Kψ(CH2N)PR]-TASP binds different types of human cells such as CD4+ T cell lines CEM and MOLT4, PHA-stimulated PBMC, and the CD4− HeLa cells (Fig. 2). In all of these cells, the cell surface binding of FITC-labeled 5[Kψ(CH2N)PR]-TASP was specific since it was prevented by the unlabeled 5[Kψ(CH2N)PR]-TASP molecule (Fig. 2, sections 1, 2, 5, and 6). Interestingly, cell surface binding of FITC-labeled 5[Kψ(CH2N)PR]-TASP was prevented by all TASP constructs active against HIV-infection (not shown) such as the 5[KPR]-TASP (Fig. 2, section 4), but not by constructs which were inactive (7) such as 5[KGQ]-TASP (Fig. 2, section 3). These results are consistent with the suggestion that the different anti-HIV TASP constructs interact with the same cell surface component since they have the capacity to competitively block the binding of the FITC-labeled 5[Kψ(CH2N)PR]-TASP to cells.

In an attempt to determine the proteinaceous nature of the cell surface component to which peptide-TASP inhibitors bind, cell surface labeling of MOLT4 cells with FITC-labeled 5[Kψ(CH2N)PR]-TASP was investigated after proteolysis with trypsin or proteinase K. As controls for proteolysis, we investigated the expression of cell surface CD26 with the mAb Ta1 and CD4 with mAbs OKT4 and OKT4A against CD4. The peak C in each section represents the corresponding control peak obtained by PE-labeled control mAb B4 (specific to CD19) for mAb Ta1, FITC-labeled MCG1 control antibody for mAbs OKT4 and OKT4A, and 0.5 μM unlabeled 5[Kψ(CH2N)PR]-TASP for p19*.

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The Higher Affinity of 5[KW(CH2N)PR]-TASP Compared with 5[KPR]-TASP to Bind the Cell Surface—5[KW(CH2N)PR]-TASP and its nonreduced counterpart 5[KPR]-TASP are potent inhibitors of HIV-1 entry and infection (7), with IC50 values in CEM cells as 0.5 and 5 μM, respectively (Table II). For further characterization of the TASP-inhibitor binding protein on the cell surface, biotin-labeled TASP inhibitors along with control TASP constructs (5[QPQ]- and 5[KGQ]-TASP) that lack activity against HIV infection, were investigated by FACS analysis (Table II; Fig. 4). Clearly, no cell surface labeling occurred with control TASP molecules. On the other hand, both biotin-labeled 5[KW(CH2N)PR]- and 5[KPR]-TASP molecules were found to bind CEM cells with 50% effective binding concentration values of 0.15 and 3.5 μM, respectively. These results illustrate that 5[KW(CH2N)PR]-TASP manifests, at least, 10-fold higher activity compared with its nonreduced TASP-counterpart for both the inhibition of HIV infection and the affinity to bind the cell surface ligand. This latter favors the hypothesis that inhibition of HIV infection is a consequence of specific binding of the TASP inhibitor to its cell surface ligand.

5[KPR]- and 5[KW(CH2N)PR]-TASP are stable in the FACS buffer. However, when incubated in serum from fetal calf or from an individual seropositive for HIV-1, 5[KPR]-TASP rapidly loses its activity (probably due to proteolysis) with a half-life of about 1 h. In contrast, 5[KW(CH2N)PR]-TASP retains more than 80% of its activity after 18 h of incubation at 37°C (Table II).

Identification of a 95-kDa Protein in Cell Extracts by Ligand Blotting Using Biotin-labeled 5[KW(CH2N)PR]-TASP—Crude extracts from CEM cells were assayed by ligand blotting using 5 μM of biotin-labeled 5[KW(CH2N)PR]-TASP. A single protein band, migrating just underneath the 97-kDa molecular mass protein marker was revealed; this 5[KW(CH2N)PR]-TASP binding protein is referred to as p95 (Fig. 5). The binding was specific since it was abolished in the presence of excess unlabeled 50 μM 5[KW(CH2N)PR]-TASP (Fig. 5B), whereas 50 or 100 μM 5[QPQ]- and 5[KGQ]-TASP had no effect (as in Fig. 5A). Under similar experimental conditions, biotin-labeled 5[KPR]-TASP construct but not 5[QPQ]- or 5[KGQ]-TASP constructs revealed p95 (data not shown).

In order to determine the molecular mass of p95 under non-denaturing conditions, cell extracts were subjected to gel filtration chromatography, and fractions were analyzed by ligand blotting. The 5[KW(CH2N)PR]-TASP binding protein eluted as a protein of molecular mass between 90 and 100 kDa (data not shown; the experimental conditions were as described under “Materials and Methods”). A small amount of an 80-kDa protein was detectable in fractions containing p95. However, since the elution profile of this 80-kDa protein was identical to that of p95, then the 80-kDa protein is most probably a degradation product of p95, which could have been generated even during SDS-PAGE. Indeed, if the degradation had occurred before gel filtration, then the elution of the 80 kDa protein would have been delayed in relation to that of p95.

Stable Complex Formation between 5[KW(CH2N)PR]-TASP and the Cell Surface-expressed p95—CEM cells preincubated with biotin-labeled control and anti-HIV TASP constructs were washed extensively, and cell extracts were purified by avidinagarose, in order to isolate any potential complexes formed between the biotin-labeled TASP constructs and cell surface proteins (“Materials and Methods”). The presence of p95 in such purified preparations was then revealed by ligand blotting using biotin-labeled 5[KW(CH2N)PR]-TASP. Under these experimental conditions, p95 was recovered when cells were preincubated with either biotin-labeled 5[KPR]- or 5[KW(CH2N)PR]-TASP. Under these experimental conditions, p95 was recovered when cells were preincubated with either biotin-labeled 5[KPR]- or 5[KW(CH2N)PR]-TASP (Fig. 6, lanes 4 and 5), but not with biotin-labeled control TASP constructs 5[KGQ]- or 5[QPQ]-TASP (Fig. 6, lanes 2 and 3). Consistent with the higher affinity of 5[KW(CH2N)PR]-TASP to bind its cell surface ligand compared with 5[KPR]-TASP construct (Table II; Fig. 4), an almost 2-fold higher amount of cell surface p95 was recovered by the reduced compared with the un-reduced TASP inhibitor (Fig. 6, lanes 4 and 5). The isolation of cell surface p95 by preincubation of cells with biotin-labeled 5[KW(CH2N)PR]-TASP was specific since it was completely abolished in the presence of an excess of unlabeled 5[KW(CH2N)PR]-TASP during the preincubation period (Fig. 6, compare lanes 5 and 7). Consistent with

**Table I**
The FITC-labeled 5[KW(CH2N)PR]-TASP binds to a cell-surface protein resistant to trypsin but sensitive to proteinase K and pronase E digestion

| Protease         | Cell surface expression | % positive cells |
|------------------|-------------------------|----------------|
| TASP-ligand      | CD4                     | CD26           |
| None             | 100                     | 100            |
| Trypsin          | 92                      | 24             |
| Proteinase K     | 15                      | 6              |
| Pronase E        | 8                       | 9              |

**Table II**
The higher activity and stability of 5[KW(CH2N)PR]-TASP compared with 5[KPR]-TASP

To calculate the IC50 values for the inhibition of HIV infection, different concentrations (0.25, 0.5, 1, 5, 10, 20, 50, and 100 μM) of each construct were added to CEM cells 15 min before the addition of HIV-1. The production of HIV (measured by the concentration of p24) was monitored at 4 days post-infection (7). The affinity to bind the cell surface ligand was assayed by FACS analysis using biotin-labeled TASP constructs (as in Figure 4). The 50% effective concentration (EC50) represents the dose of the TASP-inhibitor to reveal 50% labeling of cells, considering that the maximum mean fluorescence intensity was 100%. For the stability of the TASP inhibitors, biotin-labeled 5[KPR]- and 5[KW(CH2N)PR]-TASP constructs (at 50 μM concentrations) were incubated in decomplemented serum (heated at 56°C, 30 min) from fetal calf (FCS) and from an HIV-seropositive individual (HIV-1+ serum). After 1 and 18 h of incubation at 37°C, aliquots were tested by FACS analysis to estimate the capacity of each construct to bind the cell surface ligand; the results are presented as percent residual activity at each time point compared with that obtained with both constructs incubated under similar experimental condition but in PBS.

| TASP construct      | Inhibition of HIV infection IC50 | Affinity to bind TASP-ligand, EC50 | % Residual activity |
|---------------------|---------------------------------|----------------------------------|---------------------|
|                     |                                 |                                  | FCS, 1 h/18 h       |
| 5[QPQ]-TASP         | None                            | None                            | Not tested          |
| 5[KGQ]-TASP         | None                            | None                            | Not tested          |
| 5[KPR]-TASP         | 5 μM                            | 3.5 μM                          | 65/15               |
| 5[KW(CH2N)PR]-TASP  | 0.5 μM                          | 0.15 μM                         | 92/84               |

a No effect at 100 μM.
b No binding at 20 μM.
its lower affinity to bind p95, an excess of 5[KPR]-TASP abolished about 40–50% (Fig. 6, compare lanes 5 and 6). On the other hand, the control 5[QPO]-TASP construct had no effect (Fig. 6, lane 8).

These results, therefore, demonstrate that the biotin-labeled TASP inhibitors bind to the cell surface-expressed p95 and that this complex is stable since it could be isolated by the strong affinity of biotin to bind avidin. The 5[KPR]-TASP complex is highly stable at physiological salt concentrations but dissociates at concentrations of NaCl > 200 mM (data not shown). To confirm that p95 isolated under experimental conditions described in Fig. 6 was indeed from the cell surface, CEM cells were iodinated to label cell surface proteins, before incubation with biotin-labeled 5[QPO]- or 5[KPR]-TASP constructs (Fig. 7). Cells were then washed, extracted, and the biotin-labeled TASP-protein complexes were isolated by purification using avidin-agarose. By ligand blotting, we first demonstrated that, when cells were preincubated with biotin-labeled 5[KPR]-TASP but not 5[QPO]-TASP, then p95 was recovered after purification (Fig. 7B, lanes 2 and 3). The hypothetical degradation product of p95, the 80 kDa protein was once again detected along p95 (Fig. 7B, lane 3). Analysis of the purified preparation by SDS-PAGE and autoradiography revealed that both p95 and the 80-kDa by-product were labeled with $^{125}$I and were isolated specifically when cells were preincubated with the biotin-labeled 5[KPR]-TASP construct (Fig. 7A, lane 3). A highly $^{125}$I-labeled 140-kDa protein was found to bind avidinagarose independent of the biotin-labeled TASP constructs (Fig. 7A, lanes 1–3). The identity of this 140-kDa protein is not known. The isolation of $^{125}$I-labeled p95 from labeled cell surface proteins was also demonstrated by preincubation of cells with biotin-labeled 5[KPR]-TASP (data not shown; similar to those in Fig. 7). Consequently, the results shown in Fig. 7 provide further confirmation along those shown in Fig. 6, that a significant proportion of p95 is expressed on the cell surface and that this protein interacts specifically with 5[KPR]-TASP and related anti-HIV TASP constructs.

Comparison of the estimated amount of the p95 found in crude cell extracts (Fig. 6, lane 1) with that isolated from the cell surface (Fig. 6, lane 5) suggested that cell surface p95 could represent less than 20% of the total cellular p95. This is consistent with the low amount of p95 that we could recover in plasma membrane preparations (see “Materials and Methods”) compared with that found in cytoplasmic extracts (data not shown).

The Presence of a p95-like Protein in Different Types of Human and Marine Cells—In ligand blotting-type experiments (data not shown; experimental procedures as in Figs. 5 and 6) using cell extracts and the biotin-labeled 5[KPR]-TASP, we could demonstrate the expression of a 95-kDa protein in different types of human (MOLT4, Jurkat, HeLa, and Daudi) and murine (NIH/3T3, L929, and hydridoma) cells, similar to p95 in CEM cells.

5[KPR]-TASP Does Not Interact with HIV Proteins—Several observations indicated that 5[KPR]-TASP does not interact with HIV-1 envelope gp120-gp41 or with other viral proteins (data not shown). First, by FACS analysis, we demonstrated that FITC- or biotin-labeled 5[KPR]-TASP does not react with the cell surface gp120-gp41 complex expressed by chronically HIV-1-infected cells. Second, HIV-1 particles were not retained on an affinity column constructed with the biotin-labeled 5[KPR]-TASP bound to avidin-agarose. Third, $^{125}$I-labeled gp120 (as in Fig. 1A) or metabolically $^{125}$I-methionine-labeled HIV-1 proteins (as described in Ref. 8) were not retained on the 5[KPR]-TASP affinity column. Finally, in ligand blotting-type experiments using extracts from concentrated HIV-1 Lai particles, we demonstrated that the biotin-labeled 5[KPR]-TASP binds to the 95-kDa protein.
has suggested the participation of a cell surface protease, sim-
ilar to trypsin, referred to as trypatase TL2 (16, 35), the Fc
receptor (36), adhesion molecules LFA-1 (37–39) and ICAM-3
(40), major histocompatibility complex class I and class II mols-
enes (41–42), cellsurface antigens CD7 (43) and CD44S (44),
and finally cell surface membrane-associated components such
as heparan sulfates (45), lectins (46), and glycolipids (47). In
CD4 negative cells, galactosyl-ceramide has been shown to be
the responsible factor for binding of HIV particles (48–50). In
view of all these observations, it is conceivable that several cell
surface antigens may coordinate the complex machinery of the
membrane fusion process in which the HIV gp120–gp41 enve-
lope complex plays a key role. The requirement for some of the
individual components might depend on the cell line studied
and also might vary between the virus-cell or cell-cell fusion
processes (1, 27, 32, 39). Whatever is the case, the number of
cofactors implicated and their precise role in the HIV-mediated
membrane fusion process still remains to be determined.

More recently, convincing evidence has been provided by
several laboratories to show that the G protein-coupled recep-
tors belonging to the large family of seven-transmembrane-
spanning (7tm) cell surface proteins, such as HUMSTRF/LESTF/Fusin and CC-CKR-5, serve as species-specific
cofactors for the entry of T cell and macrophage-tropic HIV-1
isolates, respectively (51–55). Moreover, the cofactor role of
CC-CKR-5 was shown to be influenced by the structure of the
V3 loop (54). However, as yet, it is not known whether the
latter is due to a direct interaction with the V3 loop.

The results presented here demonstrate that 5[KΨ(CH₃N)PR]-TASP binds specifically to a cell surface-expressed 95-kDa
protein. The close relationship between the anti-HIV activity and the affinity to bind such a cell surface protein indicates
that the inhibition of HIV entry might be a direct consequence of a complex formation between 5[KΨ(CH₃N)PR]-TASP and
p95. Thus, p95 might be acting as a potential cofactor of CD4
during the functioning of the gp120-gp41 complex. Consistent
with this, 5[KΨ(CH₃N)PR]-TASP and related anti-HIV TASP
constructs block major functions of the gp120-gp41 complex to
initiate membrane fusion, viral entry, and apoptosis (Ref. 7; see
"Results and Discussion"). Preliminary results obtained in our
laboratory have indicated that a synthetic V3 loop peptide...
corresponding to the HIV-1 Lai gp120 sequence has the capacity to reveal p95 in ligand blotting type experiments and also form a stable complex with the cell surface-expressed p95 in CEM cells (similar to the results described in Figs. 5 and 6). As the TASP inhibitors were originally designed to mimic the conserved RD peptidomotif in the V3 loop of HIV-1 and HIV-2 isolates (7), it is plausible then to suggest that such inhibitors manifest their anti-HIV effect by blocking the potential interaction of the V3 loop with p95. It is unlikely that p95 is either Fusin or CC-CCKR-5 since these recently described cofactors have molecular masses less than 50 kDa (56, 57). Nevertheless, it will be essential to determine the implication of p95 in relation to these cofactors in the HIV envelope-mediated membrane fusion mechanism. The interaction of p95 in relation to these cofactors in the HIV envelope-medi- ated membrane fusion mechanism. 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