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Irene Zaldívar, María Ángeles Muñoz-Fernández, Balbino Alarcón and Ester San José

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Expression of a Modified Form of CD4 Results in the Release of an Anti-HIV Factor Derived from the Env Sequence

Irene Zaldívar,* María Ángeles Muñoz-Fernández, † Balbino Alarcon, 2* and Ester San José, *

We have studied the inhibitory effect of a CD4 chimera (CD4e15) on HIV replication. This chimera is retained in the endoplasmic reticulum and traps the HIV envelope precursor gp160, preventing its maturation. Retroviral expression of the chimera strongly inhibited HIV replication even when it is expressed by only a minority of the T cell population. This protective effect on bystander nontransduced cells is mediated by a soluble factor that we identified as a fragment of HIV gp120 envelope protein and accordingly, we named this factor Env-derived antiviral factor (EDAF). Biochemical and immunoreactivity data show that EDAF is comprised of the gp120 C3-C5 regions and indeed, a recombinant protein bearing this sequence reproduces the anti-HIV properties of EDAF. Surprisingly, three tryptic peptides derived from EDAF are homologous but not identical with the corresponding sequences of the HIV isolate used to generate EDAF. We propose that EDAF results from an alternative intracellular processing of the Env protein provoked by its association to CD4e15 and the selection of the best fitted Env protein sequences contained within the HIV isolate. The presence of EDAF improves the therapeutic potential of the CD4e15 gene and it opens new possibilities for antiviral treatment and vaccine development. The Journal of Immunology, 2009, 183: 1188–1196.

The therapeutic strategies currently used to combat HIV infection were developed in the early 1990s. In general, they rely upon the inhibitory influence of at least three different small molecules on one or two enzymes vital for HIV replication: reverse transcriptase and protease. The combination of these potent drugs, known as highly active antiretroviral therapy (HAART), 3 has greatly improved the prognosis for patients with HIV infection (1–3). However, HAART does not eradicate the virus, which remains latent in cellular reservoirs, implying that this therapy must be administered continually. If we also consider the severe side effects of the drugs and the emergence of HIV drug-resistant mutants (4–6), it is clearly worthwhile searching for alternative anti-HIV strategies.

Molecules that specifically block the binding of the HIV envelope protein (gp120) to its receptors, as well as the fusion process, constitute a new class of receptor-based therapeutic agents for HIV type 1 (HIV-1) infection and gp120-mediated pathogenesis (7). In addition to inhibitors of entry and fusion, gene therapy is another alternative approach to HAART. The idea of genetically modifying cells to express genes with anti-HIV activity has been proposed as a potential treatment for AIDS patients, and a steady stream of gene-based interventions using different strategies have been described in cell culture experiments (8, 9).

The life cycle and replication of HIV begins when the HIV envelope glycoprotein (gp120) recognizes the CD4 molecule and the CCR5 or CXCR4 coreceptors (10). The envelope glycoproteins are synthesized as a 160 kDa precursor in the endoplasmic reticulum (ER), and this precursor is proteolytically processed by cellular proteases in the Golgi apparatus (11) to produce mature gp120 and gp41 present in the virion. The processing of gp160 is inefficient due to the formation of intracellular complexes with CD4 and only 10–15% of the precursor molecule is processed and exported to the plasma membrane. The excess of both proteins is degraded in lysosomes (12) and in the case of CD4, by a mechanism dependent on the HIV-1 protein Vpu (13).

We have already shown that the expression of a CD4 chimera that is retained in the ER, CD4e10, inhibits HIV-1 replication (14). The interaction of gp160 with CD4e10 appears to provoke its retention in the ER, thereby preventing its maturation and export to the Golgi apparatus. This chimera is composed of the complete CD4 molecule with 10 aa of the CD3e ER retention signal appended to its C terminus. Stable expression of CD4e10 in T cells protects them from the cytopathogenic effects of HIV. Furthermore, transduction of human T lymphoblasts from seropositive individuals with retroviral particles derived from the Moloney virus inhibited the depletion of the CD4+ population. However, these studies were performed in suboptimal conditions, using a retroviral vector that did not allow the transduced cells to be followed, and with low titer retroviral supernatants (10⁴ PFU/ml). We have studied a bicistronic vector that coexpresses a longer version of the CD4e15 gene and it opens new possibilities for antiviral treatment and vaccine development.
Materials and Methods

Retroviral vectors and plasmids

The pcIE15 and pD15 constructs were obtained by cloning CD4e15 into the bicistronic retroviral vectors pCIE (pLZR-CMV-gfp) (16, 17) provided by Dr. A. Bernard (Centro Nacional de Biotecnología, Madrid, Spain) and MP91 provided by Dr. D. von Lauer (Institute for Biomedical Research, Frankfurt, Germany) (18). To express the fragments of gp120, they were cloned into the fowlpox virus vector that transduces the leader sequence (19). For some experiments that required purification, construct III was tagged at the C terminus with a stretch of 6 histidine residues.

Cells and Abs

All the CD4+ T lymphoblastic cell lines used in the infection experiments (Jurkat, PM1, and MT2) were obtained from the American Type Culture Collection. The cell clone C10 was obtained through the limiting dilution of MT2 cells transduced with the pcIE15 construct and selected for GFP expression. These cells were maintained in RPMI 1640 plus 5% heat-inactivated FBS. Peripheral mononuclear cells from healthy donors were isolated using Ficoll-Hypaque (Amersham Biosciences) that were stimulated with 1 μg/ml phytohemagglutinin (Sigma-Aldrich) and reconstituent IL-2 (100 U/ml; Roche) at 37°C for 48–72 h, and propagated in complete medium in the presence of 100 U/ml IL-2.

The polyclonal anti-gp160 antisera were provided by G. del Real (Inвестigación y Tecnología Agraria y Alimentaria, Madrid, Spain) and it was raised against the purified gp160 protein. Abs against different regions of gp120 (C1-ARP 3076, C3-ARP 3051, C4-ARP 388, C5-ARP 3221, V3-EVA 331), anti-p24 (ARP 313), and anti-p17 (ARP 3057) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The PE-coupled anti-human CD4, CD69, CD25, and CXCR4 Abs were purchased from BD Pharmingen. The H-370 anti-CD4 Ab used to probe Western blots was from Santa Cruz Biotechnology, and the Leu-3a anti-CD4 Ab labeled with APC was purchased from BD Biosciences.

HIV strains and infection

The Tropic HIV-1 strain NL4.3 (20) was provided by Dr. J. Alcamí (Instituto de Salud Carlos III, Madrid, Spain), whereas the CBL23 HIV-2 strain was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The Ba-L M tropic strain (National Institutes of Health AIDS Research and Reference Reagent Program) was grown on blood human monocytes. Infection experiments were conducted as previously described (14, 21). Viral replication was monitored by measuring the HIV-1 p24 Ag in the supernatant of the cultures by ELISA (Innogenetics) and by visualizing the cytoplasmic expression. These cells were maintained in RPMI 1640 plus 5% heat-inactivated FBS. Peripheral mononuclear cells from healthy donors were isolated using Ficoll-Hypaque (Amersham Biosciences) that were stimulated with 1 μg/ml phytohemagglutinin (Sigma-Aldrich) and reconstituent IL-2 (100 U/ml; Roche) at 37°C for 48–72 h, and propagated in complete medium in the presence of 100 U/ml IL-2.

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Cell transfections and transductions

Vectors were produced and transduced as previously described (17), and transient transfection of COS-7 and Jurkat cells was achieved by electroporation.

Activation experiments

Ag stimulation of the TCR complex was conducted by incubating the CH7/C17 derivative of Jurkat T cells at 37°C in the presence of IL-2 (100 U/ml) and CD3e15-transduced DAP murine fibroblasts (DAP-DR1) at an APC to T cell ratio of 1:1. APCs were preincubated overnight at 37°C with increasing concentrations of influenza hemagglutinin (HA) peptide 307–319 in RPMI 1640 medium supplemented with 1% FBS. The cells were stimulated for 24 h to detect CD69 and for 48 h to detect CD25. After stimulation, the cells were transferred to ice-cold PBS, washed twice, and stained with Abs against CD69 and CD25 directly labeled with PE. The samples were analyzed in a FACSCalibur (BD Biosciences) flow cytometer, and the mean fluorescence intensity was measured at each point for the green cells (CD4e15- or mock-transduced cells). The supernatants from these cultures (24 h after stimulation) were analyzed to detect IL-2 by ELISA (Cultek).

Purification of the antiviral factor by density gradients

The culture supernatant from CD4e15-transduced and HIV-infected MT2 cells was ultracentrifuged at 100,000 × g for 90 min at 4°C. The pellet was resuspended in 1.5 ml PBS and layered on a discontinuous gradient of iodixanol (OptiPrep; Invitrogen). Ultracentrifugation was performed in a 70.1 Ti rotor (fixed angle) at 250,000 × g for 90 min at 4°C, and the fractions were taken from the top of the tube.

Purification and characterization of the antiviral factor by chromatography

Size exclusion. A 250 ml Sephacryl S-200 HR column (Amersham) was calibrated with a commercial kit (Amersham), 20 ml of the antiviral supernatant was loaded onto the column and then 20-ml aliquots were collected.

Con A-Sepharose column. A total of 2 ml of antiviral supernatant was incubated with 500 μl of a Con A-Sepharose slurry (Sigma-Aldrich) and the soluble factor was eluted with increasing concentrations of α-methylmannopyranoside (Sigma-Aldrich).

Cation exchange column. The antiviral supernatant was passed through an Econo-Pac High S Cartridge (Bio-Rad). The column was washed with MOPS 20 mM (pH 7.4), and the bound proteins were eluted with increasing sodium chloride concentrations.

Immunoprecipitation and Western blot analysis

COS-7 cells were transfected by electroporation with either the empty vector (pSRo-HA) or with the pSRo-HA vector expressing a C-terminal hexahistidine tagged construct III (pSRo-HA-III-His). The COS cells were lysed for 30 min on ice in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, and 0.5% Brij 96 (Sigma-Aldrich) 48 h after transfection in the presence of protease inhibitors (1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF, and 10 mM iodoacetamide). The cleared lysates, as well as the supernatant from transfected COS cells, were precipitated overnight at 4°C with Ni-NTA agarose beads (QiaGen). The precipitates were washed and incubated with a lysate of 8 × 106 Jurkat cell lysate overnight at 4°C. The beads were then washed, boiled in Laemmli buffer, resolved on a 10% SDS-PAGE gel under reducing conditions, and transferred to nitrocellulose membranes (Bio-Rad). The membranes were subsequently incubated with anti-CD4 or anti-gp160 Abs for 1 h at room temperature, followed by peroxidase-labeled goat anti-rabbit IgG (1/7000; Pierce) for 30 min at room temperature, and Ab binding was visualized by ECL (SuperSignal West Dura Extended Duration Substrate; Pierce).

In other experiments, the purified construct III protein bound to Ni-NTA beads was eluted with 200 mM imidazole and used in competition experiments with the Leu-3a anti-CD4 Ab. Briefly, MT2 cells were incubated with a 1/10 dilution of the eluted protein III at 4°C for 30 min. Afterward, the cells were stained with Leu-3a directly labeled with APC and analyzed in a FACSCalibur (BD Biosciences) flow cytometer.

Results

Expression of CD4e15 does not affect the surface expression of endogenous CD4 or T cell activation

Because the CD4e15 chimera could potentially interact with endogenous CD4 in transduced T cells, impairing both CD4 expression at the plasma membrane and CD4+ T cell function, we assessed whether transduction of CD4e15 influenced CD4 expression. MT2 cells transduced with pCIE15, a vector coexpressing CD4e15 and GFP, expressed similar levels of surface CD4 to those of nontransduced cells or cells transduced with the empty vector (Fig. 1A). Furthermore, CD4 expression was identical in GFP-negative and GFP-positive cells. Expression of the CXCR4 HIV coreceptor was also unaltered by CD4e15 transduction (Fig. 1A).

To assess whether transduction of CD4e15 exerts a negative effect on CD4 function during T cell activation, we transduced the CD4+ CH7/C17 T cell line and followed its activation in response to a MHC class II-presented Ag. The CD25 and CD69 activation markers were induced in CD4e15-transduced cells at levels comparable to the control cells transduced with the empty vector (Fig. 1B). Furthermore, the release of IL-2 into the culture supernatant in response to Ag was not affected by CD4e15 expression (Fig. 1C). These results indicate that CD4e15 expression does not interfere with TCR-driven T cell activation.

Expression of CD4e15 exerts a protective effect on bystander nontransduced T cells

To study the potential anti-HIV effect of the CD4e15 chimera, the human MT2 and Jurkat T cell lines, as well as human blood T cell
HIV-1 infection during the experiment (Fig. 2). Protection was measured by the levels of p24 Ag in the culture supernatant of CD4+ T cell lines expressing CD4e15 or the empty vector. Furthermore, we detected a dose-dependent effect, both in terms of p24 release and of CD4+ T cell protection, when human T lymphoblasts expressing CD4e15 with different efficiencies were infected with the 1996 clinical isolate (Fig. 2C). Indeed, the percentage of CD4+ T cells recovered to the levels of uninfected cells. The strong reductions obtained in HIV replication and the complete protection of the cell population when only a minority of the cells was transduced suggests the existence of a protective bystander effect on nontransduced cells.

An alternative explanation is that the number of transduced cells was underestimated and that the percentages of cells expressing CD4e15 was higher than that of GFP-positive cells detected by flow cytometry. To exclude this possibility and to demonstrate the existence of a bystander effect, mixed cultures of CD4e15-expressing C10 cells (a cell clone from CD4e15-transduced MT2 cells) and parental MT2 cells were infected with HIV. Complete protection against HIV replication could be observed even when as few as 10% of the cell population expressed CD4e15 (Fig. 2D). This result unambiguously demonstrated that the expression of CD4e15 in a small population of T cells exerted a protective bystander effect on nontransduced cells.

Cells expressing CD4e15 and infected with HIV produce a soluble factor that prevents the replication of HIV in nontransduced cells

Because T cells acquired resistance to HIV-1 when only a few of them carried the therapeutic CD4e15 protein, a soluble factor that offers protection to untransduced cells may be released. To determine whether this might be the case and to exclude the need for cell-to-cell contact, experiments were performed in culture plates with two chambers separated by a 0.4-μm pore membrane that permits the diffusion of macromolecules but not of cells (Boyden chambers). Transduced and untransduced cells were infected and incubated in these chambers (as shown in Fig. 3), and HIV replication in the MT2 cells in the lower chamber was strongly reduced when CD4e15-expressing cells were grown in the upper chamber rather than parental MT2 cells (Fig. 3). This result was independent of the viral isolate (NL4.3, HIV-1; CBL23, HIV-2) or of the T cell line (MT2, PM1) used. Therefore, CD4e15-expressing cells appear to release an antiviral factor that diffuses into the lower chamber, protecting nontransduced cells from HIV infection.

The antiviral factor is a medium-sized, positively charged and particle-associated glycoprotein

To characterize the antiviral factor, we first determined whether the antiviral factor was constitutively secreted by CD4e15-expressing cells or whether it was released upon infection. We found that the antiviral factor was present in the supernatant of MT2 cells transduced with CD4e15 and infected with HIV but not in that of nontransduced or noninfected cells (Fig. 4A). Hence, both CD4e15 expression and HIV infection are required for release of this antiviral factor. Furthermore, the antiviral factor appears to be released late into the supernatant as the maximal activity was not detected until 24 h postinfection (Fig. 4B).

To characterize the antiviral factor, we transduced the CD4e15-expressing constructs pCIE15 or pD15. Although the efficiency of transduction was not always 100% (Fig. 2A), we tested the susceptibility of the transduced cell population to HIV infection. Transduced cell populations were infected with the T-tropic (X4) strain NL4.3 or with the M-tropic (R5) Ba-L strain of HIV-1 at low multiplicity of infection (MOI, 10−3 or 10 ng, respectively). When HIV replication was measured by the levels of p24 Ag in the culture supernatant, transduction of CD4e15 protected both cell lines against HIV-1 infection during the experiment (Fig. 2B). Interestingly, there was a 10,000-fold reduction in p24 release from MT2 cells (Fig. 2B), even if only 33% of the cell population was transduced (Fig. 2A). A similar effect was also observed in Jurkat cells when only 56% of the cells were transduced (Fig. 2, A and B). A protective effect was also detected when MT2 cells were infected with the primary ME46 isolate (group M, subtype B), the HIV-2 CBL-23 laboratory strain and the chimeric SIV-HIV virus SHIV 86.9 strain (data not shown), suggesting that CD4e15 expression has a broad ranging anti-HIV effect. The protection exerted by CD4e15 was also stronger than expected in primary T cells infected with the X4 and R5 strains, given that only 25% of the cells were transduced (Fig. 2, A and B). Furthermore, we detected a dose-dependent effect, both in terms of p24 release and of CD4+ T cell protection, when human T lymphoblasts expressing CD4e15 with different efficiencies were infected with the 1996 clinical isolate (Fig. 2C). Indeed, the percentage of CD4+ T cells recovered to the levels of uninfected cells. The strong reductions obtained in HIV replication and the complete protection of the cell population when only a minority of the cells was transduced suggests the existence of a protective bystander effect on nontransduced cells.
that the antiviral factor could be a slow-diffusing large molecule or particle. Indeed, when the culture supernatant was ultracentrifuged at 100,000 x g and the antiviral activity concentrated in the pellet rather than in the cleared supernatant, suggesting that the antiviral factor is, or is associated to, a particle (Fig. 4).

We tested the antiviral activity of the factor after fractioning the supernatant through a cation exchange column, a lectin column (Con A) and a size exclusion column. Maximal antiviral activity was detected in fractions that corresponded to a positively charged protein eluted with 100 mM NaCl (Fig. 4E), to a glycosylated protein eluted with 200–500 mM -methyl mannoside (Fig. 4F), and to a protein of 25–43 kDa (Fig. 4G).

Identification of the antiviral factor as a fragment of gp120

We used two different methods to identify the antiviral factor based on the properties described above. In the first approach we performed a series of ultracentrifugations based on the fact that the antiviral factor concentrates in the pellet (Fig. 4D). After subjecting the culture supernatant of HIV-infected clone C10 to centrifugation at 100,000 x g, the pellet was resuspended and centrifuged on an iodixanol density gradient, where the antiviral activity concentrated in the intermediate density fractions (fractions 5–7, Fig. 5A). As a control, the culture supernatant of nontransduced HIV-infected MT2 cells was fractioned following the same protocol and no protective effect was detected in any of these fractions (Fig. 5A).

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Unfortunately, silver staining after SDS-PAGE of the principal protective fractions from C10 cells did not reveal specific protein bands (data not shown) and thus, the antiviral factor was further purified by ultracentrifuging the fractions from the first density gradient with antiviral activity in a second iodixanol density gradient. The fractions from this second gradient were tested for antiviral activity and analyzed by SDS-PAGE and silver staining. Maximal protection was coincident with the presence of a 32–35 kDa protein doublet in fraction 10 (Fig. 5B). When both bands were trypsin digested and analyzed by manual interpretation of mass spectrometry (MS/MS spectra, the only reliable sequence obtained corresponded to a heptapeptide (STWNNGTR). This peptide had 100% sequence identity with the C3-V4 boundary of gp120 from 93 HIV isolates (Fig. 5C and data not shown).

The second approach to purify the antiviral factor involved the serial application of the three chromatography techniques already tested: size exclusion, lectin affinity, and cation exchange. After this last column, the protective and a nonprotective fraction were resolved by SDS-PAGE and stained with SYPRO-Ruby. Four apparently specific bands were detected in the protective fraction (Fig. 5D), of which three corresponded to contaminants (keratins and trypsin). The relevant band (band 2) yielded two peptides of interest that were identified by BLAST analysis as highly homologous to sequences present in region C3 of gp120, or at the V3-C3 boundary in 75 HIV isolates (Fig. 3E, and data not shown).

Because the two different approaches coincided in identifying peptides corresponding to sequences present in the gp120 envelope protein of HIV, the antiviral factor produced by cells expressing the CD4e15 chimera and infected with HIV appeared to be a fragment of gp120. To demonstrate this hypothesis, we first determined whether the gp120 fragment identified by MALDI-TOF was only produced by CD4e15-transduced cells. We found that a ~35 kDa protein recognized by an anti-gp120 polyclonal antiserum was concentrated in the intermediate fractions of iodixanol gradients obtained from culture supernatants of CD4e15-transduced MT2 cells infected with HIV (Fig. 6A, bottom). However, this protein was not detected in the culture supernatant of nontransduced MT2 cells infected with HIV, even though a thick band corresponding to the full-length gp120 was present in the heaviest fractions (Fig. 6A, top). The 35-kDa gp120 fragment was also detected in the culture supernatant of both PM1 and Jurkat cells transduced with CD4e15 (Fig. 6B). At this point, we named this gp120 fragment from Env-derived antiviral factor (EDAF).

To study the composition of EDAF in terms of the regions of gp120 that make it up, a panel of region-specific mAbs was assayed in immunoblots after fractionation on iodixanol gradients. We found that although an anti-C1 Ab reacted with gp120 but not with EDAF, an anti-C3 Ab and an anti-C4 Ab reacted equally well with both proteins. An anti-V3 mAb reacted with the upper part of the gp120 fragment but not with the lower part, suggesting that the doublet observed in some gels (e.g., Figs. 5B and 6A, bottom) corresponded to fragments of gp120 in which the V3 sequence was present or absent (see Fig. 6C for a summary of the Ab reactivity of EDAF). Because EDAF pelleted at 100,000 × g, we thought it might form part of a defective HIV particle, lighter than the full virus (Fig. 4D). However, Abs against the p24 capsid protein and the p17 matrix protein did not react with the fractions that contained EDAF (Fig. 6C). Furthermore, the HIV genome was not detected in these fractions by PCR (data not shown). These results, suggested that EDAF is not associated to viral particles or pseudoparticles and thus, the appearance of EDAF in the pellet may indicate that some of these gp120 fragments could form aggregates.
A recombinant protein corresponding to the C3-C5 sequence of gp120 mediates the antiviral effects of EDAF

According to the information gathered from MS/MS and the immunoreactivity displayed, EDAF must contain the C3 and C4 but not the C1 sequences, and it may or may not contain V3. Considering these restrictions and the size of EDAF (~35 kDa), we inserted four gp120-derived constructs into an expression vector, with or without the V3 region and the gp41 fusion peptide, to determine whether any of them possessed antiviral activity (Fig. 7A). The fact that the Abs used against the C5 sequence was not informative (Fig. 6C), prevented a more precise definition of the recombinant protein. The four constructs were transiently transfected into COS cells and the culture supernatants of these cells were assayed for antiviral activity. Unlike the other constructs, construct III presented significant anti-HIV activity (Fig. 7B), which was also manifested when construct III was transiently transfected in Jurkat T cells before they were infected with HIV (Fig. 7C). Hence, the sequence comprising C3-C5 appears to have anti-HIV activity reinforcing the idea that the antiviral factor produced by HIV-infected CD4+ expressing T cells is a fragment of gp120 consisting of the last third of the protein sequence. The difference in size and mobility of the recombinant protein III (Fig. 7B) when compared with EDAF (Fig. 6, A and B) could be due to differences in glycosylation or to the presence of C-terminal sequences from region C5 in construct III not present in EDAF.

By comparing the sequences of gp120 and the EDAF fragment (C3-C5) we ventured that the soluble factor might inhibit the interaction of CD4 with gp120. This idea is consistent with the fact that the majority of the amino acids of gp120 that interact with CD4 are present in EDAF (Fig. 7D). To test this hypothesis, recombinant protein III purified from both the culture supernatant and lysate of transfected COS cells was incubated with Jurkat T cell lysates, showing clearly that CD4 (from the Jurkat cell lysate) associated with the recombinant protein III (Fig. 7E). Furthermore, purified construct III also blocked the staining of MT2 cells with the Leu-3a CD4 mAb known to compete with gp120 for CD4 binding (Fig. 7F) (22). Together, these data show that EDAF binds CD4 and they suggest that it interferes with HIV infection by competing with the gp120-CD4 interaction.

Discussion

Because the endoproteolytic maturation of the Env precursor protein is a crucial step in the production of viral particles (23), our initial aim was to develop a strategy in which the stable expression of a CD4 chimera permanently retained in the ER blocks
maturation of gp160. Indeed, this key step in the viral cycle is an increasingly attractive target for inhibitor design (24). Because the replication of different HIV strains was strongly inhibited in the presence of CD4ε15 (including laboratory strains, clinical isolates, X4 and R5 strains), it would appear that this construct has good potential as a therapeutic gene. However, more interestingly the CD4ε15 exerts a protective bystander effect on nontransduced cells. Indeed, the protective bystander effect of CD4ε15-expressing cells is mediated by the release of a soluble antiviral factor upon infection with HIV.

Several antiviral factors have been described, including chemokines like CCL5 (25) and cytokines such as IFN-α (26). An antiviral factor produced by activated CD8⁺ T cells (CD8 antiviral factor) was first described several years ago (27), although its molecular characterization is still pending (28, 29). Another anti-HIV soluble factor is produced by CD4⁺ T cells from nonprogressing seropositive patients (30) and it is also thought that T cells infected with attenuated virus, such as vif-defective HIV, release a soluble antiviral factor (31). The antiviral factor produced by CD4ε15-expressing T cells is released after HIV infection, suggesting that it is either induced or encoded by the viral genome. Using two different biochemical approaches to purify the antiviral factor, we have identified this factor as a fragment of gp120. According to the sequence of three tryptic peptides identified, its immunoreactivity using domain-specific Abs, and the size of the factor (~35 kDa), the fragment must correspond to a polypeptide included in the last third of the gp120 sequence. Furthermore, transfection of a recombinant protein comprising the C3-C5 sequence of gp120 confers antiviral activity. These results positively identify the antiviral factor as a gp120 fragment, which we have named EDAF.

The C3-C5 fragment comprises most of the outer domain of gp120 and part of the bridging sheet (32–34). Significantly, 21 of the 26 aa of gp120 that make contact with CD4 are present in EDAF. Therefore, most CD4-interacting regions in gp120 are present in EDAF and thus, we predict that it could interfere with recognition of the CD4 cell receptor by HIV. Indeed, this factor binds to CD4 and it competes with Leu-3a, an anti-CD4 Ab that recognizes the gp120-binding site in CD4 (22).

**FIGURE 6.** Immunological characterization of EDAF. A, Iodixanol density fractions obtained as in Fig. 5A were separated in a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and incubated with a polyclonal anti-gp120 antiserum that identified the 35-kDa gp120 fragment. The reactivity of the anti-gp120 antiserum with fractions bearing the gp120 present in nontransduced infected MT2 cells is also shown, although these cells do not produce the 35-kDa gp120 fragment. B, The same as A with culture supernatants of PM1 and Jurkat cells transduced with CD4ε15 and infected with HIV. C, Reactivity in Western blots of protecting fractions with Abs specific for different HIV proteins and gp120 regions.

**FIGURE 7.** Evaluation of the antiviral activity of recombinant EDAF. A, Organization of the variable and constant regions of the immature gp160 glycoprotein, and the positions of gp120 corresponding to the three tryptic peptides identified are indicated. The gp120 regions contained in constructs I-IV are shown. Construct I- V3-C5 sequence; construct II- V3-C5 sequence and fusion peptide; construct III- C3-C5 sequence; construct IV- C3-C5 sequence and fusion peptide. B, Constructs I-IV or empty vector (d) were transfected into COS cells and the culture supernatant was collected 48 h postinfection and tested in a protection assay. Data presented are the mean and SD of an experiment performed in triplicate. Level of protein expression in immunoblots with an anti-CD4 Ab was illustrated (inset). C, Jurkat cells were transiently transfected with either the empty vector (control) or with construct III. At 48 hours after transfection, cells were infected with isolate NL4.3 at a MOI of 0.1 and 0.5 and the p24 Ag release was measured. D, Ribbon diagram representing the tertiary structure of the gp120 monomer. The C3-C5 sequence is highlighted in cyan, whereas the CD4 contact sites are shown in red. E, Construct III bearing a hexahistidine tag or an empty vector (d) were transfected into COS cells, and the cell lysates and culture supernatants were incubated with Ni-NTA beads. Afterward, the beads were incubated with a Jurkat cell lysate and the precipitate was analyzed in Western blots probed with an anti-CD4 Ab and eluted with 200 mM imidazole. F, Purified protein III obtained as in E and eluted with 200 mM imidazole was preincubated with MT2 cells on ice before the Leu-3a APC anti-CD4 Ab was added for 30 min at 4°C. The cells were analyzed by flow cytometry. MT2 cells treated with purified protein III (red line), MT2 cells treated with empty vector (blue line), and untreated MT2 cells (gray line) are shown. G, Alignment of the three tryptic peptides from EDAF with the gp120 sequence of the NL4.3 isolate. A dash indicates identity and an asterisk indicates homology.
The mechanism by which EDAF is produced in cells expressing the CD4e15 chimera is more puzzling. Because CD4e15 interacts with the gp160 precursor in the ER and this interaction inhibits the normal processing to gp120 and gp41 (14), it is likely that the gp160-CD4e15 complex is processed abnormally. This processing perhaps takes place in a compartment other than the trans-Golgi network where gp160 proteolysis is believed to occur (35). Our preferred hypothesis is that by complexing to gp160, CD4e15 drives this protein into a proteolytic compartment, perhaps the lysosome, where gp160 is degraded except for the regions that most closely contact CD4e15, which would be sterically protected. Accordingly, EDAF would result from the fingerprinting of CD4e15 onto gp120. Indeed, the observed variability in the size of purified EDAF and the presence of more than one protein band could be the consequence of the mechanism by which EDAF is generated, resulting from partial proteolytic cleavage. Interestingly, although a BLAST analysis of the three tryptic peptides used to identify the antiviral factor shows 100% identity with gp120 sequences of different HIV isolates, their sequences do not exactly match that of the NL4.3 isolate, the HIV strain used to produce the factor. Indeed, sequencing a cDNA representing the bulk population of our isolate, the HIV strain used to produce the factor, shows 100% identity with gp120 sequences of different HIV isolates. However, if our hypothesis is correct, one might venture that only the gp120 sequences best fitted to interact with CD4e15 during proteolytic attack would be those that form EDAF. Accordingly, EDAF would result from the selection of gp120 sequences encoded by viral variants present in the NL4.3 population that are capable of interacting with CD4e15, even after removal of the C1-V3 sequences.

Although we initially set out to study the prospect of expressing CD4e15 as a gene therapy approach against HIV, these experiments led us to identify an antiviral factor derived from the gp120 sequence. The existence of this antiviral factor increases the potential benefits of using CD4e15 in gene therapy or to raise vaccines. Alternatively, the EDAF antiviral factor could be used directly as an inhibitor to treat HIV infection or to raise vaccines. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. Microbiol. Rev. 59: 63–93.


