Inhibition of CD4 Translation Mediated by Human Immunodeficiency Virus Type 1 Envelope Protein in a Cell-free System*

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The human immunodeficiency virus type 1 (HIV-1) employs a number of complex strategies to interfere with the translation, stability, and subcellular localization of its specific cellular receptor CD4. To define better the mechanisms of inhibition of CD4 expression, we used a rabbit reticulocyte lysate in vitro system, in which cDNAs derived from HIV-1-infected cells were used to generate mRNA for the Tat, Vpu, and gp160 envelope proteins that were translated together with CD4-encoding mRNA. In the presence of microsomal membranes, we observed that cotranslation of Env mRNA resulted in a dose-dependent inhibition of CD4 translation. This effect was enhanced further when an mRNA-encoding Vpu in addition to Env mRNA was utilized. However, the activity of Vpu was mostly post-translational, since translation of Vpu alone, but not Env, was able to destabilize CD4 molecules presynthesized into microsomes. The Env-mediated inhibitory effect was specifically targeted at CD4 and did not affect the synthesis or stability of the CD8 molecule. Interestingly, mutated CD4 species, with a 20-fold lower affinity for HIV-1 Env than wild-type, were less sensitive to cotranslational inhibition. Our report identifies the envelope as the HIV-1 protein responsible for down-regulation of CD4 translation. We further propose a mechanism whereby direct interactions between gp160 and nascent CD4 molecules can cause interference with and premature termination of CD4 protein elongation.

Infection of CD4+ cells by the human immunodeficiency virus type 1 (HIV-1) generally leads to cell surface depletion of the CD4 receptor (for review, see Ref. 1). This down-regulation of CD4 is largely dependent on the formation of intracellular complexes between CD4 and the gp160 viral envelope precursor (2–5). The generation of such CD4-gp160 complexes in the endoplasmic reticulum of infected cells has been shown to impair the maturation and ultimate transport to the cell surface of both proteins, while simultaneously increasing their stability and steady-state levels (6–8). This mechanism of interference is tightly regulated by the intracellular CD4:gp160 ratio. Indeed, when amounts of CD4 are superior or equal to those of gp160, it is conceivable that formation of these complexes ultimately shuts off production of infectious progeny. More efficient control of cell surface CD4 expression could be targeted at the biosynthesis or stability of this molecule, both to decrease the number of complexes and to allow gp160 maturation. Such specialized mechanisms have been shown to target both the transcription of the CD4 gene (9–12) and the stability of the CD4 protein (13–15). Although the processes leading to altered CD4 transcription remain elusive, recent studies have identified the Vpu and Nef proteins of HIV-1 as major effectors of post-translational degradation of CD4 (13, 16–19). Vpu induces CD4 degradation in the endoplasmic reticulum and preferentially targets CD4 molecules trapped in complexes with the gp160 envelope precursor (14, 19), whereas Nef has been shown to induce CD4 internalization from the cell surface followed by lysosomal degradation (for reviews, see Refs. 1 and 20). Both Vpu and Nef act through specific recognition sequences located in the cytoplasmic domain of CD4 (21–27).

An additional level of CD4 down-modulation was shown to result in reduced amounts of CD4 proteins recovered from cells expressing HIV-1 (9, 10, 28, 29). It was then postulated that HIV-1 infection caused a decrease in the rate of CD4 biosynthesis. (28). However, the effect of HIV-1 infection on CD4 translation could not be clearly addressed because of the use of in vivo models in which both transcriptional and post-translational levels of CD4 down-regulation contributed to the overall decrease in CD4 abundance. In addition, both the mechanisms and viral proteins involved in a presumed inhibition of CD4 translation remained unknown. To examine directly the translational level of CD4 down-regulation following HIV-1 infection, the present study employs a cell-free translation system, consisting of rabbit reticulocyte lysates and microsomal membranes in which translation is qualitatively and quantitatively controlled at the mRNA level. Similar systems have been used in the past to dissect mechanisms of virus-mediated inhibition of cellular protein translation (30–32). Our study focuses on the effect of the HIV-1 envelope and Vpu proteins on CD4 translation, both because of the affinity of gp160 for CD4 and because they are both membrane-anchored proteins translated in the same subcellular compartment as CD4. In addition, both Vpu and gp160 translated in cell-free translation systems, similar to that employed here, were shown to be biologically functional (23, 33, 34).

We now report that the translation of bicistronic mRNA species encoding both the Vpu and gp160 proteins of HIV-1...
potently inhibited CD4 translation in the presence of canine pancreatic membranes. The use of mRNA species that had been mutated to inactivate the Vpu open reading frame (ORF) further showed that gp160 was the major contributor to the co-translational inhibitory effect. Translation of Vpu alone had little effect on CD4 synthesis; rather, Vpu induced post-translational degradation of CD4, as reported previously (23). To examine whether translational inhibition observed with the envelope was consistent with competition at the level of initiation, CD4 was translated in the presence of mRNA encoding the Tat protein. Even though the tat mRNA initiates translation with high efficiency (35–37), it could not inhibit CD4 translation to a level comparable to that of the envelope. Rather, we found that inhibition of CD4 translation occurred at the level of elongation and was dependent on specific interactions with the envelope protein. In agreement with this finding, translation of control molecules such as the structurally related CD8 was not affected by the envelope protein. Taken together, our data show that in addition to the previously reported transcriptional and post-translational levels of CD4 down-regulation, HIV-1 has evolved an additional level of interference by specifically acting on the translation of its receptor. The specificity of this translational inhibition for CD4 is explained by the fact that it requires direct interactions between the envelope protein and nascent CD4 molecules.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids and in Vitro Transcription—pT4B (kindly supplied by Drs. Paul Maddon and Richard Axel, Columbia University, New York) and pT8F1 (obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health, and contributed by Dr. Richard Axel) (38) were linearized with BamHI and used as templates to synthesize CD4 and CD8 mRNAs. The BamHI-EcoRI restriction fragment containing the complete cDNA of the CD4-M1 envelope binding mutant (kindly provided by Dr. R. Sekaly) (39) was inserted into the pGEM4 vector (Promega, Madison, WI) linearized with BamHI and EcoRI. The pGEM4Z-CD4-M1 expression vector was linearized with BamHI for the purpose of in vitro transcription, and the Sp6 RNA polymerase was used to generate the 1.8-kilobase CD4-M1 mRNA. The use of BamHI removed the 3′-noncoding region of CD4. Homologous and CD8 mRNAs contained the 5′-leader sequences necessary for efficient translation into membranes. pGEM15E, pGEM15EU−, and pGEM14E were generated by subcloning the BssHII-XhoI restriction fragments from pNL15E, pNL15EU−, and pNL14E (kindly supplied by Dr. Barbara Felber, National Cancer Institute, Frederick, MD) (37), into pGEM3Z (Promega). This subcloning strategy removed most of the first noncoding exon from the original cDNA and the ORF of nef at the 5′-end, pGEM15E and pGEM15EU− were linearized with XhoI and used as templates to synthesize either the bicistronic 15E mRNA, which encodes both Env and Env, or the mutated 15EU− mRNA, which lacks a functional vpu initiation codon but is otherwise identical to 15E. Alternatively, pGEM15E was linearized with NdeI to produce a monocistronic mRNA, encoding only Vpu. When linearized with NdeI, pGEM14E gave rise to an mRNA that contained reading frames for each of the first exons of tat and rev as well as the entire vpu ORF. As expected, this mRNA only produced Tat and low amounts of Vpu when subjected to in vitro translation (37). All mRNAs were synthesized using the Sp6 RNA polymerase in the presence of 0.5 mM ATP, CTP, UTP, and m7GpppG (Promega). To ensure efficient capping of the mRNAs, the concentration of GTP was decreased to 0.125 mM. RNA was purified as described previously (36), deprotected by a nuclease-treated rabbit reticulocyte lysate (Promega), and used as templates for the synthesis of the respective mRNAs to allow post-transcriptional processing of membrane-associated proteins. Before in vitro translation, both cellular and viral mRNAs were heated at 67 °C for 10 min to unfold secondary structures that would eventually affect the efficiency of mRNA translation (41). Reactions were assembled as recommended by the supplier in the presence of 20 μCi of [35S]methionine (ICN Biochemicals). After a 1-h incubation at 30 °C, samples containing microsomes were centrifuged at 12,000 rpm for 3 min at 4 °C. Membrane pellets were rinsed by recentrifugation in buffer (25 mM Hepes, pH 7.2, 65 mM KCl) and lysed in electrophoresis sample buffer. Alternatively, lysis was in 20 mM Tris-HCl, pH 8, 120 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate for purposes of immunoprecipitation. Samples were then analyzed in their entirety on 12% SDS-polyacrylamide gels. Following electrophoresis, gels were soaked in EN3Hance (NEN Life Science Products), dried, and exposed to X-Omat AR films. Gels were also exposed to Phosphor B1 screens, and the bands corresponding to CD4 were quantified by scanning of the screens in a GS-290 molecular imager (Bio-Rad).

Time Course of CD4 Stability—40 μl of canine pancreatic membranes was assembled in a single in vitro translation reaction in the presence of 6 μg of CD4 mRNA. After a 30-min incubation at 30 °C, the membranes were centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was removed and the membranes rinsed in 25 mM Hepes, pH 7.2, 65 mM KCl. The membrane pellet, loaded with CD4, was then resuspended in a new in vitro translation mix, and equal amounts of this master mix were aliquoted into samples containing the same copy number of either 15E, 15EU−, 15E NdeI, 14E NdeI mRNAs, or no mRNA as control. Samples were incubated at 30 °C, and aliquots were collected for analysis at 15-min intervals over the course of a total 1-h incubation. Membranes were centrifuged at 12,000 rpm for 5 min at 4 °C, lysed in electrophoresis sample buffer, and analyzed for protein content on 12% SDS-polyacrylamide gels. After autoradiography, the gels were exposed to Phosphor B1 screens and the bands of interest quantified as described above.

RESULTS

In Vitro Expression and Identification of env, tat, and vpu—To study inhibition of CD4 translation in the presence of the HIV-1 envelope protein, mRNAs similar to those expressed in HIV-infected cells were used (37). Expression vectors were obtained as described under “Experimental Procedures” and subjected, after linearization at the indicated sites (arrows), to in vitro transcription using the Sp6 promoter. The proteins produced by the different mRNAs, after in vitro translation in the presence of microsomes, are indicated on the right (parentheses indicate low production of protein).
the effect of vpu and env on CD4 synthesis, in a nef-independent fashion. The cellular and viral mRNAs shown in Fig. 1 were used to program rabbit reticulocyte lysates in the presence of canine pancreatic membranes (microsomes). After translation for 1 h at 30 °C, the microsomes were separated from the supernatant by centrifugation and their protein content analyzed directly by SDS-PAGE.

The results of Fig. 2 show that both CD4 and CD8 proteins as well as the envelope precursor were converted efficiently to their respective glycosylated forms of 58, 34, and 160 kDa, when microsomes were present. In addition, cleavage of the gp160 envelope precursor to mature gp120 was also detected. Both Vpu and Tat were translated efficiently. Fractionation of the membrane and supernatant fraction further showed that vpu is primarily associated with the microsome fraction. In contrast, the Tat protein was more abundant in supernatants (data not shown). The ratios at which each of gp160, Vpu, and Tat were synthesized by the three different mRNAs correspond to the positions and relative strengths of their respective AUGs, indicating the fidelity of our system to ribosomal scanning models (42). In the case of the 15E mRNA, gp160 was produced by leaky scanning of the vpu AUG codon and was thus less abundant than in the case of 15EU mRNA which only contained the env AUG (Fig. 2). As expected, the strong tat AUG present in the 14E mRNA potently inhibited expression of the downstream ORFs, dramatically reducing expression of both Vpu and Env proteins (Fig. 2) (37).

To address the effect of vpu on CD4 translation, the pGEM 15E plasmid was linearized with NdeI and used to produce an mRNA containing only the vpu ORF (Fig. 1). The pGEM 14E plasmid was also linearized with NdeI to produce an mRNA that encoded both Tat and Vpu, but the Tat AUG that is upstream of the Vpu AUG is a very strong initiation codon. Therefore, synthesis of Tat is initiated with such high efficiency that production of the downstream Vpu is compromised. These two truncated mRNAs, along with the complete 15E and 15EU− mRNAs, were translated in the presence of canine pancreatic membranes. The membranes were separated from the supernatant by centrifugation and the identity of the proteins synthesized by each mRNA assessed by immunoprecipitation (Fig. 3). Antibodies directed against Env, Vpu, or Tat were able to immunoprecipitate each relevant protein, indicating that proper synthesis and post-translational modifications had occurred. As expected, the 15E mRNA generated each of gp160 and gp120, as well as abundant quantities of Vpu, consistent with the location of the vpu ORF at its 5'-end. The 15EU− mRNA contained a mutated vpu AUG and produced correspondingly higher levels of Env protein caused by the absence of trans-
lational initiation at the vpu ORF. Removal of the env ORF, by linearizing both the 15E and 14E plasmids with NdeI, led to the complete disappearance of both gp160 and gp120 and provided us with mRNAs that only encoded Vpu and Tat, respectively.

**Inhibition of CD4 Synthesis in the Presence of the 15E and 15EU**

Viral mRNAs—Bicistronic mRNA molecules, which contain the vpu ORF upstream of the env ORF (37), similar to the 15E mRNA in this study, are known to produce the Vpu and gp160 proteins, both implicated in CD4 down-modulation (6, 7, 19, 23). The 15E mRNA and its vpu-defective counterpart, 15EU−, were tested for their effect on CD4 synthesis, when cotranslated in the presence of CD4 mRNA at different ratios. After 1 h at 30 °C, the microsome fraction of the in vitro translation reaction was recovered by centrifugation. To ensure the quantitative recovery of all of the proteins synthesized during the in vitro reaction, the membrane fraction was resuspended in electrophoresis sample buffer without further manipulation, and the samples were analyzed in their entirety by SDS-PAGE (Fig. 4A). The amount of CD4 glycoprotein generated in the absence of viral mRNA was proportional to the amount of CD4 mRNA employed, indicating that none of the mRNA concentrations used saturated the in vitro translation system (Fig. 4A, lanes 1–4). Cotranslation of these same CD4 mRNA dilutions in the presence of constant amounts of 15E mRNA led to a profound reduction in CD4 synthesis (Fig. 4A, lanes 6–9), even when a 4-fold excess of CD4 mRNA over 15E mRNA was present. At the same time, translation of each of Vpu and gp160 was unaffected, indicating that the decrease of CD4 translation observed was not caused by a generalized inhibition of protein translation (compare lanes 5 and 6–9).

**Fig. 4. Inhibition of CD4 translation by the 15E and 15EU**

Panel A, different molecular ratios of CD4 mRNA relative to env mRNAs were cotranslated for 1 h in the presence of canine pancreatic membranes and [35S]methionine. The membrane fraction was then separated by centrifugation, and the labeled proteins were analyzed by electrophoresis on 12% polyacrylamide gels. The positions of the cellular and viral proteins are indicated on the left. Panel B, the intensities of the bands corresponding to CD4 in panel A were calculated after exposure of the gels to Phosphor screens. The results are expressed as the ratio of levels of CD4 generated when translated in the presence of viral mRNA relative to control levels obtained when the same amounts of CD4 mRNA were translated alone (lanes 1–4). The levels of CD4 were individually compensated for the different background levels in each lane by subtracting the value reported for a identical area drawn above the band corresponding to CD4. The results were expressed as a percentage of CD4 levels present in control lanes and were plotted as a function of the CD4/15E or CD4/15EU− molecular ratios used. Numbers from 6 to 24 on the x axis refer to the corresponding lane numbers in panel A.
Gp160 and CD4 were translated with similar efficiency in the in vitro system despite the higher intensity of the gp160 band observed in Fig. 4A. Because the intensity of the bands is directly proportional to the amount of [35S]methionine incorporated in each protein, a measure of the translation efficiency must take into account the relative amount of methionine residues in each protein. The proportion of methionine residues in gp160 is approximately 1.7-fold higher than in CD4, which probably accounts for the higher intensity of the gp160 band.

Efficient inhibition of CD4 synthesis was also observed when increasing concentrations of 15E mRNA were cotranslated with a constant amount of CD4 mRNA (Fig. 4A, lanes 10–14), even when CD4 mRNA was present at a 20-fold excess (lane 10). Inhibition of CD4 synthesis increased with the amount of 15E mRNA and was maximal when both mRNAs were present at equimolar concentrations (lane 14). However, this last result should be interpreted cautiously, since, in this case, a general diminution in translation of both CD4 and viral proteins was observed. This may be caused by the large amount of RNA which was present in this lane, leading to reduced efficiency of the cell translation system.

Because the Vpu protein was shown to induce CD4 degradation in vitro (23), we asked whether the inhibitory effect of the 15E mRNA could be attributed, in part or in whole, to the expression of the Vpu protein. We therefore performed similar experiments using the 15EU−, vpu-defective, mRNA. As shown in Fig. 4A, lanes 15–24, CD4 synthesis was inhibited efficiently by the expression of Env alone. However, this did not occur to the extent observed when both Vpu and gp160 were generated (Fig. 4A, lanes 5–14). This indicates that both proteins contribute to the overall decrease in CD4 abundance.

We found that the percentage of CD4 relative to control was not always the same when equimolar concentrations of CD4 mRNA and gp160 mRNA were used (Fig. 4A, compare lane 7 with 14, 17 with 24). This is probably due, in part, to the fact that lanes 14 and 24 contained twice as much total RNA as that present in lanes 7 and 17, causing a reduced overall efficiency of translation. It is also likely that the total amount of Env-encoding mRNA is an important factor in addition to the ratio of the different types of mRNA in the system.

The bands corresponding to CD4 in Fig. 4A were quantified by densitometry scanning using a molecular imager. Inhibition of CD4 synthesis was expressed as the ratio between CD4 intensity in the presence of viral mRNA and the intensity observed when the same amount of CD4 mRNA was used in the absence of viral mRNA. For each individual lane, the intensity of CD4 was compensated for the background generated by the translation of mRNAs encoding the envelope protein. As shown in Fig. 4B, the combined effect of the envelope protein and Vpu led to approximately 70% inhibition of CD4 translation, even when CD4 mRNA was in a 2–4-fold excess over the 15E message (Fig. 4B, lanes 8, 9, and 13). In the presence of equimolar or excess of 15E, the inhibition of CD4 translation reached more than 95% (Fig. 4B, lanes 6 and 7). A 10–20-fold excess of CD4 mRNA was necessary to synthesize more than 80% of the CD4 present in the control (lanes 10 and 11). Densitometric analysis of CD4 synthesized in the presence of the 15EU−mRNA showed that the removal of the vpu ORF restored about 10% of the CD4 proteins at all ratios used. Impairment of CD4 translation remained very efficient, attesting that the env ORF was the major effector of CD4 down-modulation at the translational level.

Envelope-mediated Inhibition of CD4 Translation Is Not Caused by Post-translational Degradation or Competition for Initiation of Translation—To address directly the role of Vpu in the inhibition of CD4 translation observed in Fig. 4, the pGEM

15E vector was linearized with NdeI to produce an mRNA encoding Vpu but not gp160 (15E NdeI). This mRNA was used in cotranslation experiments with CD4 mRNA and yielded far less inhibition of CD4 synthesis than seen with either vpu- or tat-encoding mRNAs (Fig. 5A, lanes 1–14). Quantitation of CD4 in Fig. 5A shows that the effect of the Vpu protein on the rate of CD4 synthesis is modest (Fig. 5B, lanes 6–14). A maximal inhibition of close to 50% was reached only when the CD4 and Vpu-expressing mRNAs were present in

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similar amounts (Fig. 5B, lanes 6 and 7, 13 and 14). Thus, translation of gp160 is apparently the major cause of CD4 translational repression, and Vpu plays a relatively minor role in this regard.

We also asked whether nonspecific competition for translation initiation might be the cause of the inhibitory effect observed in Fig. 4. Toward this end, CD4 was cotranslated with the 14E NdeI mRNA, for which 5'-tat ORF possesses a strong signal for initiation of translation (36, 42). Consequently, 14E NdeI essentially expresses the Tat protein and small amounts of Vpu (Fig. 1). The same mRNA ratios used in Fig. 4 were applied to the 14E NdeI and CD4 mRNAs that were cotranslated in vitro in the presence of microsomes. Fig. 5A, lanes 15–24, shows that either constant or increasing concentrations of 14E NdeI mRNA had little effect on CD4 translation. Densitometric analysis of CD4 bands indicated a maximum 53% inhibition of CD4 translation when a 2:1 excess of 14E NdeI mRNA was used (Fig. 5B, lane 16). At this same ratio, the 15EU mRNA that only expresses Env inhibited CD4 synthesis by 90% (Fig. 4B, lane 16). The small effect of the 14E NdeI RNA on CD4 translation at high concentrations is likely because of the presence of the small amounts of Vpu produced from this RNA (Fig. 5B, lanes 15–19, 23 and 24). Thus, competition at the initiation level is not responsible for the diminished CD4 translation observed with 15E and 15EU mRNAs. Because the AUGs that precede the env and vpu ORFs are weaker than that of tat, it is unlikely that such competition could have accounted for the inhibition observed when CD4 was cotranslated with these mRNAs.

Vpu but Not gp160 Acts at the Post-translational Level by Decreasing CD4 Stability—Of all the ORFs used in this study, vpu has the weakest AUG compared with the consensus sequence (35). Nonetheless, cotranslation of vpu and CD4 mRNAs inhibited CD4 protein accumulation to a greater extent than tat mRNA. As stated, Vpu has been reported to decrease CD4 stability both in vivo and in vitro (14, 19, 23). To ask whether the modest drops in CD4 synthesis, observed with the 15E and 15EU NdeI mRNAs, were attributable to post-translational CD4 destabilization, we subjected microsomes containing fixed amounts of presynthesized CD4 to a second round of in vitro translation with the 15E, 15EU−, 15E NdeI, or 14E NdeI mRNA. Samples were collected every 15 min, and the rate of CD4 recovery was assessed by SDS-PAGE analysis of membrane fractions (Fig. 6A). Incubation of the CD4-loaded microsomes for 1 h at 30 °C did not affect CD4 stability during the chase time (lanes 1–4). However, a marked alteration in CD4 stability was seen at 1 h of chase after translation of the Vpu-expressing 15E and 15E NdeI mRNAs (lanes 5–8 and 13–16). This effect was post-translational and probably led to complete degradation of CD4, since no band corresponding to a CD4 cleavage product could be detected. This decrease in CD4 pool size was unlikely to be due to a poor recovery of microsomes, since increasing amounts of both gp160/gp120 and Vpu were detected during the 1-h chase period. Rather, the post-translational destabilization of CD4 was due to the presence of Vpu, since 15EU mRNA had no effect on CD4 stability (Fig. 6A, lanes 9–12). Densitometric analysis of the CD4 bands revealed a slight increase in abundance after 1 h of chase in the absence of viral mRNA (Fig. 6B) probably because of the presence of trace amounts of CD4 mRNA from the first translation step. A similar CD4 profile was obtained in the presence of 15EU mRNA (Fig. 6, A and B, lanes 9–12), confirming that the decreased levels of CD4 obtained after cotranslation with 15EU mRNA did not involve post-translational alterations. In contrast, translation of both 15E and 15E NdeI mRNAs did affect CD4 stability. Expression of Vpu alone (CD4–15E NdeI) or in the presence of the envelope (CD4–15E) induced a similar 2-fold decrease in CD4 abundance after 1 h of chase (Fig. 6B). This indicates that although the envelope protein enhances Vpu-mediated degradation of CD4 in vivo (14, 19), it is not required for degradation. Translation in this protocol of 14E NdeI mRNA gave rise to a modest 15% degradation of CD4 (Fig. 6B, CD4–14E NdeI), a result likely due to the low levels of Vpu produced by this mRNA.

Taken together, these results show that neither competition for initiation of translation nor post-translational degradation can account for the profound inhibitory effect of the HIV-1 envelope protein on CD4 synthesis. The fact that gp160 neither altered CD4 stability nor enhanced the destabilizing effect of Vpu rather indicates that its effect is at the translational level.

Mutation of the Envelope Binding Site Protects CD4 from Translational Inhibition—To define better the molecular mechanisms involved in envelope-mediated inhibition of CD4 translation we examined whether direct interactions between the two proteins were involved. To this end, we examined the effect of envelope synthesis on the translation of a mutated form of CD4 impaired in gp160/gp120 binding (CD4-M1) (39). The CD4 and CD4-M1 mRNAs were translated for 1 h in the presence of constant or increasing amounts of 15EU mRNA, the membrane fraction was separated by centrifugation, and its protein content analyzed on 12% SDS-PAGE (Fig. 7). As shown above, the envelope protein produced by the 15EU mRNA generated a marked reduction of CD4 abundance compared with synthesis enacted in the absence of 15EU mRNA (Fig. 7A). Quantitation of the bands corresponding to CD4 revealed a dose-dependent decrease in abundance similar to that observed in Fig. 4 (Fig. 7C, CD4). In contrast, synthesis of CD4-M1 mutant was less affected than wild-type CD4 by the presence of the 15EU mRNA (Fig. 7, B and C, CD4-M1). Since CD4 and CD4-M1 mRNAs share the same 5’-region, this differential susceptibility cannot be explained by differences in initiation of translation. Moreover, the wild-type and mutated CD4 proteins are synthesized with similar efficiency in the absence of viral mRNA and have similar mobility patterns on SDS-PAGE, indicating that elongation of translation as well as post-translational folding were not affected by the M1 mutation (Fig. 7, A and B). Since the M1 mutation altered neither the synthesis, folding, nor glycosylation of CD4, the resistance of CD4-M1 to translational inhibition is likely due to the removal of the gp120/gp160 binding site. When increased concentrations of CD4-M1 mRNA were present, the modest decrease in synthesis of gp160 is probably a nonspecific effect, caused by competition for the translational apparatus.

Env Does Not Impair CD8 Translation—Our results suggest that inhibition of CD4 translation is dependent on specific interactions with the envelope protein. This raises the possibility that the Env-mediated events reported here are specific for CD4. To address this question directly, we translated a CD8-encoding mRNA in the presence of either the 15E or 15EU mRNAs. CD8 was chosen as a control because it is related to CD4 from both a structural and evolution standpoint, as both are members of the immunoglobulin-like superfamily.

When the same molecular ratios of cellular and viral mRNA used in Fig. 4 were used to translate CD8 in the presence of either 15E or 15EU mRNA, no inhibitory effect was seen in regard to accumulation of CD8 protein (Fig. 8). In contrast, the use of constant amounts of each of 15E and 15EU mRNAs (lanes 5–9 and 15–19) led to diminished gp160 levels, concomitant with the presence of higher concentrations of CD8 mRNA in the system. The modest increase in levels of CD8 generated in the presence of 15E and 15EU mRNA may be a nonspecific effect (compare lane 3 with lanes 11–13 and 17–19). Hence,
unlike the case with CD4, CD8 mRNA was translated efficiently in a dose-dependent manner and independently of the presence of either 15E or 15EU. Only at very high concentrations of cellular and viral mRNAs did we observe a decrease in CD8 protein levels (Fig. 8, lanes 14 and 24), pointing to a sharing of the translational machinery by these mRNAs rather than inhibition by either of them. This result further confirms that gp160 acts in a specific manner to inhibit CD4 translation.

**DISCUSSION**

The first objective of our study was to examine whether the decrease in immunoprecipitable CD4, observed after HIV-1 infection, could be attributed to a specific inhibition of CD4 translation rather than to the combined effects of decreased CD4 mRNA, degradation mediated by the Nef or Vpu proteins of HIV-1, and a general down-regulation of host-cell protein synthesis.

The use of an *in vitro* translation system allowed us to study translational aspects of CD4 down-regulation by controlling the levels of both cellular and viral mRNAs from each of a qualitative and quantitative standpoint. The viral mRNAs used were derived from authentic messages produced during the course of HIV-1 infection (37). However, they did not contain the double-stranded TAR structure normally present in the 5'-untranslated region of all HIV mRNAs (43). This elimination was necessary, since the TAR structure has been shown to activate the double-stranded RNA-dependent protein kinase, an event that ultimately leads to a general diminution of
cellular protein synthesis (43, 44). However, this phenomenon does not lead to a shutdown of protein translation in HIV-1-infected cells, since both the cellular TAR RNA-binding protein (45) and viral Tat (46) can counteract the negative effects of TAR on protein translation. Since neither TAR RNA-binding protein nor Tat was present in the system, the removal of TAR structures from the viral mRNAs allowed us to measure specific rather than general inhibition of CD4 translation. The use of canine pancreatic membranes permitted quantification of newly synthesized cellular and viral proteins, since subcellular relocation or secretion did not occur in microsomes as happens in functional cells. In vitro translation in the presence of microsomes yielded both cellular and viral proteins that were mature and immunoprecipitable by relevant specific antibodies (Figs. 2 and 3).

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This system allowed us to show that CD4 translation is inhibited in the presence of HIV-1 mRNAs that are monocistronic and bicistronic for gp160 and Vpu/gp160, respectively (Fig. 4). These results confirm that translational inhibition of CD4 synthesis has functional relevance and can be quantified in vitro. We further showed that translation of gp160 alone (15EU mRNAs) was both necessary and sufficient to cause effective diminution of CD4 translation. We have also shown the involvement of Vpu in CD4 diminution in this system. Translation of the bicistronic 15E mRNA yielded a more significant impairment in CD4 abundance than that obtained with 15EU Vpu-counterpart mRNA (Fig. 4). The role of Vpu was further demonstrated by cotranslating CD4 with monocistronic 15E NdeI mRNA, which encodes only Vpu (Fig. 5). Although limited, the effect of 15E NdeI translation on CD4 abundance was somewhat more pronounced than the effect of the 14E NdeI mRNA bearing the strong tat AUG.

We thus assessed whether Vpu might act alone or together with gp160 to impact on CD4 stability. Translation of the 15E NdeI mRNA in membranes containing presynthesized CD4 yielded a 50% degradation of the latter protein after 1 h (Fig. 7). These results are in agreement with studies showing that Vpu can induce CD4 degradation in in vitro systems in the absence of other viral proteins (23). More importantly, we did not observe decay of presynthesized CD4 in the presence of the 15EU mRNA. This clearly indicates that in contrast to Vpu, the envelope protein acts at the level of CD4 translation to decrease levels of CD4 produced.

A number of animal viruses have been shown to alter host-cell mRNA translation to allow preferential synthesis of viral proteins (31, 32, 47, 48). In these cases, overall rather than specific synthesis of individual cellular proteins is affected. Since HIV-1 has also been reported to inhibit cellular protein synthesis (29, 49, 50), we had to rule out a generalized inhibitory effect of 15EU mRNA in terms of the CD4 results presented here. As a control, we therefore cotranslated CD8 mRNA in the presence of the same concentrations of 15E and 15EU mRNAs that had been used in cotranslation of CD4. No effect on CD8 synthesis was seen (Fig. 7), except when vastly elevated quantities of 15E or 15EU mRNA were used. Thus, cotranslated in the presence of constant or increasing amounts of 15EU mRNA. In vitro translation was performed for 1 h at 30 °C in the presence of microsomal membranes. The membrane fraction in its entirety was analyzed by SDS-PAGE and autoradiography. The positions of the cellular and viral proteins are shown on the left. Panel C, the intensities of the bands corresponding to CD4 in panels A and B were calculated after exposure of the gels to Phosphor screens. The percentage of CD4 levels relative to control lanes were calculated and plotted as a function of the CD4/15EU or CD4-M1/15EU molecular ratios used as described for Fig. 4. Numbers from 6 to 13 on the x axis refer to the corresponding lane numbers in panels A (CD4) and B (CD4-M1).
CD8 but not CD8 translational inhibition is specifically driven by HIV-1 mRNAs that express the env gene.

This gp160-mediated effect could conceivably have affected either initiation or elongation of CD4 translation. However, translation of the 15E mRNA did not alter CD4 stability, suggesting a specific effect at the translational level. We assessed the possibility that either specific or nonspecific competition for the translation apparatus might exist between cellular and viral mRNAs. Were nonspecific competition for initiation of translation to take place, then an mRNA bearing a strong AUG should have had the same effect on CD4 translation as 15E mRNA. However, translation of the 14E NdeI mRNA, containing the strong tat AUG (36), showed no detectable inhibition of CD4 translation (Fig. 5). The fact that the 15EU\(^-\) mRNA, which possesses a weak env AUG, profoundly inhibited CD4 synthesis is further evidence that competition between strong and weak AUGs at the initiation level did not play an important role in our system. The fact that the env AUG is weak does not rule out its potential role in inhibition of CD4 translation at the initiation level. However, the 15E NdeI mRNA, defective in gp160 expression, but not the env sequences surrounding the initiation codon, caused a very limited decrease in CD4 abundance. Because this decrease could be fully attributed to the post-translational degradation of CD4 by Vpu, encoded by the 15E NdeI mRNA, specific inhibition of CD4 initiation by the env AUG is unlikely.

Since inhibition of CD4 translation is both specific and dependent on microsomes, in which CD4 and gp160 are in close proximity, at least a portion of this inhibition must be due to direct interaction between nascent CD4 and gp160. This suggests that the gp160 protein, rather than env-encoding mRNA, acts by impairing CD4 elongation. Because the gp160 binding site is located at the NH\(_2\) terminus of CD4 (i.e. V1 loop) and is nonglycosylated (51, 52), it may be able to bind to gp160 very soon after being generated and during CD4 elongation. Thus, mature gp160 in microsomes could conceivably bind to nascent CD4 peptides to prevent such elongation. We examined this possibility by using a mutant CD4 molecule, CD4-M1, with greater than a 20-fold reduction in affinity for gp120. The CD4-M1 mutant had been derived by substituting human CD4 residues located in the V1 domain with corresponding residues of mouse CD4, an approach that preserves both protein conformation as well as functional interaction with class II major histocompatibility complex molecules (39). No differences were seen between the wild-type and M1 forms of CD4 after in vitro translation and SDS-PAGE analysis (Figs. 2 and 3), indicating that CD4-M1 underwent normal translation and SDS-PAGE analysis (Figs. 2 and 3), indicating that CD4-M1 underwent normal translation and extensive folding before a functional CD4 binding site is formed (56). It is thus unlikely that CD4 could exert similar cotranslational inhibition of envelope synthesis. This is further confirmed by the lack of effect of CD4 on envelope synthesis in our in vitro system.

This is the first report of a mechanism whereby a virus specifically impairs the translation of its specific cellular receptor. Similar mechanisms could conceivably apply to the translational down-regulation of cellular receptors by other viruses. However, inhibition of CD4 translation by HIV-1 relies on two main requirements that may not be fulfilled in other cases. These are the unusually high affinity of the envelope glycoprotein for CD4 and the capacity to interact with a very discrete region of the receptor that is present and functional before the completion of CD4 translation.

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