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SHORT COMMUNICATION

Rapid degradation of CD4 in cells expressing human immunodeficiency virus type 1 Env and Vpu is blocked by proteasome inhibitors

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Human immunodeficiency virus (HIV) type 1 encodes three genes, Vpu, Env and Nef, that decrease cellular CD4. Vpu and Env act cooperatively to accelerate degradation of CD4 in the endoplasmic reticulum. Here we report that Vpu/Env-induced CD4 degradation is inhibited by lactacystin, a specific inhibitor of the proteasome, and by other proteasome inhibitors, but not by non-proteasome protease inhibitors. We also note that Vpu has amino acid sequence homology with a segment of IκB known to be involved in proteasome-mediated degradation, suggesting that HIV-1 could have transduced cellular sequences to enhance down-regulation of CD4.

Retroviral infection frequently leads to loss of cell surface virus receptor as a result of formation of an intracellular complex between viral envelope and receptor or down-regulation of the cellular receptor by other mechanisms. Three human immunodeficiency virus type 1 (HIV-1) genes are directly involved in down-regulation of the CD4 cell receptor for HIV, suggesting that down-regulation is under strong selection. These HIV gene products are Env, which binds to the extracellular portion of CD4, Vpu, which binds to the cytoplasmic tail of CD4, and Nef, which accelerates the turnover of cell surface CD4 (for review, see Bour *et al*., 1995 *a*).

It has been shown in a transient expression system that the half-life of CD4 is in the order of minutes in cells expressing HIV Env and Vpu (Willey *et al*., 1992 *b*). Because the proteasome pathway has been implicated in the degradation of proteins with very short half-lives, we wished to determine if this pathway was involved in Vpu/Env-mediated down-regulation of CD4.

Lactacystin (LC), *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) and *N*-acetyl-L-leucinyl-L-leucinyl-L-normethyional (ALLM) are small molecule inhibitors of the proteasome (Omura *et al*., 1991; Rock *et al*., 1994; Fenteany *et al*., 1995). While ALLN and ALLM also inhibit other cellular proteases, LC is reported to be specific for the proteasome, covalently modifying the 20S proteasome subunit and having no effect (at 100 μM concentration) on other cellular proteases including calpain, cathepsin B, chymotrypsin, trypsin and papain (Fenteany *et al*., 1995). To see if these drugs would inhibit Vpu/Env-mediated down-regulation of CD4, we first explored their effects on a HeLa-CD4 cell line, described below, that stably expresses HIV Rev, Vpu and Env (Fujita *et al*., 1996).

HeLa cells that express high levels of CD4 were transduced with a retroviral vector (pKF41.2) (Fujita *et al*., 1996) that contains the *neo* gene and an internal cytomegalovirus (CMV) immediate/early promoter followed by HIV-1 *rev*, *vpu* and *env*. The *env* in this vector encodes a fusion-negative variant of Env. Clones of HeLa-CD4 cells transduced by pKF41.2 were found to consist of two distinct population of cells: cells with reduced surface CD4 (low surface CD4 cells) and cells with the same amount of surface CD4 as untransduced cells (high surface CD4 cells) (Fujita *et al*., 1996). When high and low surface CD4 cells were separated by FACS, each cell type generated a mixture of high and low surface CD4 cells after a few days in tissue culture, indicating that the amount of surface CD4 was controlled by physiological factors in this system. Vpu appeared to be necessary for down-regulation of CD4 in this system since: (i) the low surface CD4 cells contained more intracellular Vpu than the high surface CD4 cells, and (ii) HeLa L cells transduced by a variant of pKF41.2 encoding a truncated form of Vpu (U35; Willey *et al*., 1992 *b*) did not have the low surface CD4 subpopulation (Fujita *et al*., 1996).

Because only about 20% of cells were low surface CD4, we attempted to increase the proportion and extent of down-regulation of CD4 in this system by modifying HIV Env. Attachment of a six amino acid ER-retrieval signal (SSKKTN) (Gaynor *et al*., 1994) to the C terminus of gp41 increased the proportion of low surface CD4 cells to about 50% and lowered surface CD4 in these cells to near background levels. One of these HeLa-CD4 clones expressing dilysine-tagged Env (clone K6-C2) was used in the following experiments.

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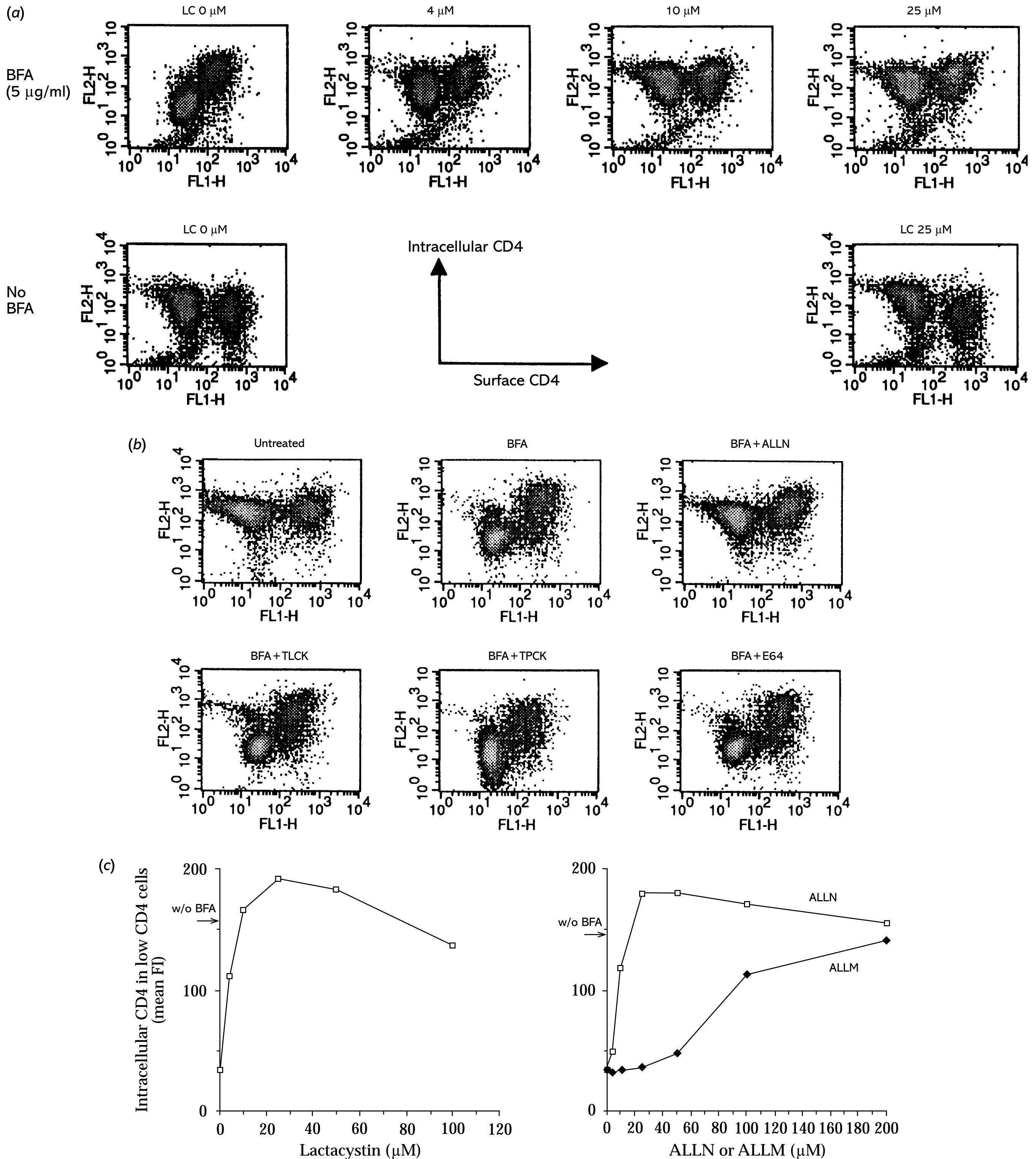

Fig. 1. Lactacystin and ALLN inhibit Vpu-induced rapid degradation of CD4, whereas the non-proteasome inhibitors TLCK, TPCK and E64 do not have this effect. (*a*) LC was added to HeLa-CD4 cells expressing Rev, Vpu and dilysine-tagged Env (clone K6-C2, see text) 40 min prior to the addition of BFA (5 μg/ml). After treatment with both LC and BFA for 6 h, cells were stained for surface CD4 with FITC-OKT4 (*x*-axis) and intracellular CD4 with PE-OKT4 (*y*-axis). (*b*) As (*a*) except that ALLN (50 μM), TLCK (50 μg/ml), TPCK (13 μg/ml), or E-64 (100 μM) were used instead of LC. (*c*) Mean fluorescent intensity of the PE-signal in low CD4 cells plotted against the concentration of inhibitors.

After preincubation with LC for 40 min to block proteasome activity, K6-C2 cells were treated with LC and brefeldin A (BFA) (5 µg/ml) for 6 h. BFA traps HIV Env, Vpu and CD4 in the ER and accelerates the HIV Env/Vpu-mediated degradation of CD4 (Willey *et al*., 1992*b*). Cells were stained for surface CD4 with FITC–OKT4 and intracellular CD4 with PE–OKT4 as described previously (Fujita *et al*., 1996). Fig. 1(*a*) shows a representative flow cytometry analysis of surface and intracellular CD4 in cells treated with BFA and LC. Untreated cells (Fig. 1*a*, lower left panel) consisted of two distinct populations of cells differing in the amount of surface CD4, as we reported for other cell lines using this vector system (Fujita *et al*., 1996). As in the clone previously reported (Fujita *et al*., 1996) the low surface CD4 cells of clone K6-C2 contained about 2–3-fold more Vpu that high surface CD4 cells (data not shown). BFA caused a 5–6-fold drop in intracellular CD4 in the low surface CD4 subpopulation (Fig. 1*a*, upper left-hand panel). This indicates that BFA accelerated degradation of intracellular CD4 as reported in a transient expression system (Willey *et al*., 1992*b*). The fact that BFA lowered intracellular CD4 in the low surface CD4 K6-C2 cells suggests that the dilysine-tagging of Env did not, by itself, lead to complete trapping of CD4 in the ER. BFA also caused a 2–3-fold rise in intracellular CD4 in the high surface CD4 cells; this is most likely explained by continued synthesis of CD4 and lack of Vpu-induced degradation of CD4 despite blocked transport to the cell surface. The rise in intracellular CD4 in the high surface CD4 cells contrasts with the drop in intracellular CD4 in the low surface CD4 cells.

LC reversed the BFA-induced drop in intracellular CD4 in the low surface CD4 cells in a dose-dependent fashion with 50% maximum effect at a concentration of ~ 4 µM (Fig. 1*a*, and 1*c*, left panel). The high surface CD4 subset of cells serves as an internal control in this experiment and shows that the LC effect is specific to the cells in which CD4 degradation is accelerated. Similar results were obtained for ALLN and ALLM (Fig. 1*c*), although half-inhibitory concentrations were ~ 9 µM and ~ 80 µM, respectively. These values correspond well with the reported relative and absolute activities of LC, ALLN and ALLM in inhibiting the proteasome (Rock *et al*., 1994; Fenteany *et al*., 1995) and are well below the concentration of LC (100 µM) reported not to affect other cellular proteases (Fenteany *et al*., 1995). We also tested protease inhibitors TLCK, TPCK and E64, which act on non-proteasome proteases; these inhibitors did not reverse the rapid degradation of CD4 (Fig. 1*b*, lower panels).

Another way to study CD4 down-regulation is through metabolic labelling/pulse-chase analysis. Such experiments are uninformative in cell lines containing both high and low surface CD4 cells because there is no way to distinguish effects on CD4 in the two subpopulations. We therefore turned to a transient transfection system in which HeLa cells were transfected with pNCRVE-IRES-CD4, a murine retroviral vector expressing HIV *rev*, *vpu* and *env* from a CMV promoter followed by an internal ribosome entry site (IRES) and the CD4 gene.

pNCRVE-IRES-CD4 was derived from pKF41.2 by inserting an IRES and the coding sequence for CD4. The IRES was amplified from pJZ308 (a gift from J. Zhang, University of Kentucky, USA) using oligonucleotides GCTGCAG <u>ATCGAT</u>TCACTATAGGGAGACC (+2795) and AAA-CCC<u>ATCGAT</u>*ACGCGT*CATATGATCATCGTGTTTTTC (−3361). *Cla*I sites (underlined) and a *Mlu*I site (italics) were added to facilitate cloning. The amplified IRES was digested with *Cla*I and inserted at a unique *Cla*I site downstream of the *env* gene in pKF41.2. The CD4 gene was excised from T4-pMV7 (Maddon *et al*., 1986) by digesting with *Eco*RI and *Bam*HI, and a *Mlu*I adapter was ligated to the blunted ends. The *Mlu*I fragment containing CD4 was inserted into the pKF41.2-IRES construct at the unique *Mlu*I site downstream of the IRES. The resulting pNCRVE-IRES-CD4 plasmid (25 µg) was used to transfect 10^6 HeLa cells using the calcium phosphate method. At approximately 16 h post-transfection, cells were metabolically labelled with 200 µCi [^{35}S]Met/Cys (Tran^{35}S-label, ICN) for 30 min, and then chased in DMEM–10% FCS; an aliquot of cells was lysed at each time-point as described (Lenburg & Landau, 1993). CD4 was immunoprecipitated with 1 µl of rabbit antiserum against CD4 (Deen *et al*., 1988) and analysed by 8·5% SDS–PAGE as described (Lenburg & Landau, 1993).

In the presence of 25 µM ALLN, CD4 had a half-life of more than 3 h, compared to about 1 h in the absence of ALLN (Fig. 2*a*, *c*). This half-life of CD4 in the absence of ALLN is comparable to that previously reported using a similar experimental system with HIV LTR-based expression vectors (Willey *et al*., 1992*a*). In contrast, in cells transfected with pCMV-CD4, a vector expressing CD4 alone (Fig. 2*b*), the half-life of CD4 was more than 4 h and was unaffected by ALLN. Thus, the ALLN effect was specific to cells expressing HIV Vpu and Env in which the half-life of CD4 is shortened. LC (25 µM) similarly increased the half-life of CD4 to about 3 h (Fig. 2*d*). Since BFA was not used in these experiments, the LC and ALLN effects in blocking CD4 degradation are not an artifact of BFA treatment. As another negative control, the LC experiment was repeated with a plasmid identical to pNCRVE-IRES-CD4 except that it contained a non-functional mutant version of *vpu* (U35; Willey *et al*., 1992*b*). In cells transfected with this *vpu* mutant version of pNCRVE-IRES-CD4, the half-life of CD4 was more than 3 h and was not prolonged by LC (Fig. 2*d*). Thus, the LC effect was only seen when CD4 degradation was accelerated by Vpu.

A novel species immunoprecipitated by anti-CD4 antibody with an apparent molecular mass of 48 kDa was conspicuous in cells treated with ALLN or LC (Fig. 2*a*, *c*) This could represent a partial degradation product of CD4 that is stabilized by ALLN or LC. A similar degradation intermediate was recently reported for CMV-induced proteasome-mediated degradation of the MHC class I heavy chain (Wiertz *et al*., 1996).

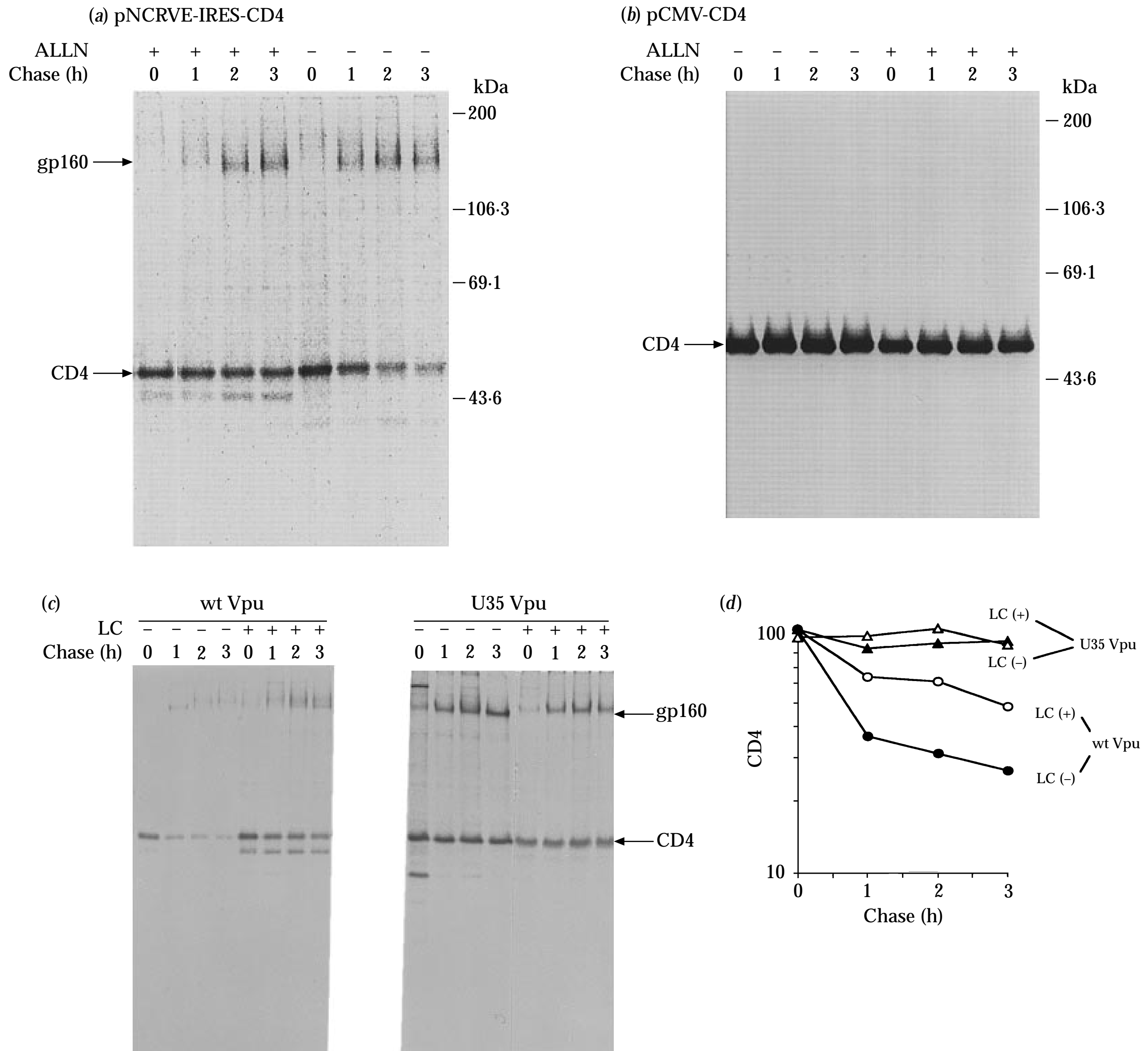

Fig. 2. ALLN and LC inhibit CD4 degradation in HeLa cells transiently expressing HIV Rev, Vpu, Env and CD4. HeLa cells were transfected with pNCRVE-IRES-CD4 (*a*, *d*) or pCMV-CD4, which expresses only CD4 (*b*). Transfected cells were either treated (+) or not treated (−) with 25 μM ALLN (*a*, *b*) or LC (*d*). Forty min later, cells were metabolically labelled for 30 min and chased for up to 3 h in the presence (+) or absence (−) of inhibitors. CD4 in the cell lysates was immunoprecipitated with rabbit antiserum against CD4 and analysed by 8·5% SDS–PAGE. Radioactivity of bands corresponding to the 54 kDa species of CD4 were quantified with a phosphorimager and normalized so that 100% equals the amount of CD4 present immediately after the pulse (*c*, *d*).

Fig. 2(*a, d*) also shows the expected cross-immunoprecipitation of HIV Env by anti-CD4 antibodies. The slight increase in HIV Env cross-immunoprecipitated by anti-CD4 antibodies with increasing time of chase is a consistent observation and may indicate that the intracellular Env–CD4 complex forms over a period of a few hours.

To test whether Vpu forms a complex with CD4 in the presence of proteasome inhibitors, labelled cells were lysed with a digitonin-lysis buffer and the complex was immunoprecipitated with the anti-CD4 antibody. At the 0 h and 1 h chase time-points, Vpu was detected in the anti-CD4 immunoprecipitate in cells treated with ALLN or LC; in the absence of the inhibitors a small amount of Vpu was detected in the CD4 immunoprecipitate at the 0 h time-point but none was detected at the 1 h time-point. This confirms that CD4 and wild-type Vpu form a physical complex (Bour *et al.*, 1995 *b*).

Given our observation that Vpu/Env-mediated CD4 degradation was decreased by proteasome inhibitors, we looked for sequence similarities between Vpu, CD4 and other proteins known to be degraded by the proteasome pathway

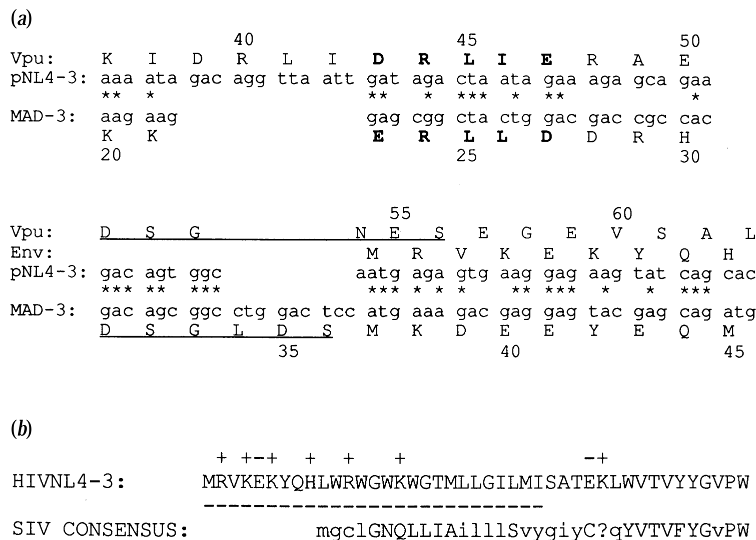

Fig. 3. (*a*) Nucleotide and amino acid sequence similarity between HIV-1 Vpu/Env and IκB. Nucleotide sequences of the pNL4–3 clone of HIV-1 (Adachi *et al*., 1986) and the MAD-3 clone of IκB (Haskill *et al*., 1991) are aligned. Identical bases are indicated by asterisks. The gap after K-22 of MAD-3 corresponds to the DRLI repeat in Vpu. The gap after G-53 of Vpu gives better identity between the two nucleotide sequences than the alignment without this gap. (*b*) The signal peptide of HIV-1 Env but not SIV Env contains charged amino acids near the N terminus. An amino acid alignment of the Env signal peptide (underlined) of the pNL4–3 clone and an SIV consensus is shown. Capital letters indicate amino acids conserved among SIV clones. A question mark indicates more than three different amino acids are seen among clones. Charged amino acids are indicated by + or −.

(Fig. 3). IκB, an inhibitory protein that binds to the transcription factor NFκB, is ubiquitinated and degraded through the proteasome pathway. Two amino acid motifs in IκB are known to play important roles in this process: (i) a pair of serines separated by three amino acids [(positions 32–36 in the MAD-3 clone of IκB (Haskill *et al*., 1991)] which become phosphorylated prior to proteasome degradation (Chen *et al*., 1995), and (ii) two lysines which are candidate sites for covalent attachment of ubiquitin about 10 amino acids upstream of the phosphoserines (Scherer *et al*., 1995). Vpu contains a six amino acid diserine motif DSGNES (positions 51–56) that is identical in four positions and has one conservative E → D change compared to the diserine motif (DSGLDS) in IκB (Fig. 3*a*, underlined). This motif is completely conserved in 14 isolates of HIV-1 (Chen *et al*., 1993). We note that the two serines in this motif are phosphorylated by casein kinase II (Schubert *et al*., 1994) and mutations of these serines in Vpu block the rapid degradation of CD4 (Schubert & Strebel, 1994), just as mutations in the homologous serines in IκB block proteasome-mediated degradation of IκB. The diserine motif in Vpu lies in a loop between two predicted alpha-helical regions on the cytoplasmic side of membrane-associated Vpu (Schubert *et al*., 1994). Thus, it is in a position to interact with proteasome proteins in the cytoplasm.

Nine amino acids upstream of its diserine motif, IκB contains the sequence ERLLD (Fig. 3*a*, bold); in the same relative position Vpu contains a closely related, repeated sequence DRLI/DRLIE (Fig. 3*a*, bold). Both the diserine motif and the DRLIE motif are absent from the U35 truncated version of Vpu that fails to accelerate degradation of CD4.

The cytoplasmic tail of CD4, which is involved in binding to Vpu (Bour *et al*., 1995*b*), contains 40 amino acids including four lysines (at positions 411, 417, 418 and 428) (Maddon *et al*., 1986) that are potential sites for attachment of ubiquitin. It is possible that interaction between the cytoplasmic portions of Vpu and CD4 brings together a diserine proteasome degradation signal in Vpu and target ubiquitination sites in CD4. In preliminary experiments, we have not been able to detect ubiquitinated CD4 in lysates of cells treated with ALLN. However, not all proteasome-degraded proteins are ubiquitinated (Murakami *et al*., 1992).

Vpu appears to be a recent evolutionary development as it is not present in simian immunodeficiency virus (SIV) and HIV-2, which are closely related to HIV-1. HIV-1 Env proteins have more than one order of magnitude higher affinity for CD4 than those of HIV-2 or SIV (Ivey-Hoyle *et al*., 1991). Strong interaction between HIV-1 Env and CD4 inside cells interferes with maturation of Env (Willey *et al*., 1992*a*); this could provide selective advantage to virus variants capable of down-regulating intracellular CD4. Concomitant with acquisition of stronger CD4 binding, HIV could have transduced cellular sequences from IκB or related diserine motif-containing genes in order to facilitate proteasome-mediated degradation of CD4, in much the same way that transforming retroviruses have transduced oncogenes. Alternatively, the sequence similarity between Vpu and IκB could result from convergent evolution.

Vpu is encoded by the same mRNA that encodes Env, and the part of *vpu* that contains the sequence similarity to IκB overlaps the 5′ end signal sequence of HIV-1 *env* (Fig. 3*a*). Interestingly, the signal sequence of HIV-1 Env is atypical when compared with other retroviral Env sequences by virtue of a cluster of charged residues at the N terminus (Fig. 3*b*); this could be a consequence of selection for a function in the overlapping domain of Vpu.

We have shown that three proteasome inhibitors block rapid degradation of CD4 induced by BFA in a stable cell line and in cells transiently transfected with a vector encoding CD4

plus HIV Vpu/Env. The relative and absolute activities of these inhibitors on CD4 degradation parallel their reported activities as proteasome inhibitors. One of the drugs (LC) reportedly does not affect other cellular proteases (Fenteany *et al.*, 1995). Lysosome inhibitors were also found not to affect HIV Vpu/Env-induced rapid degradation of CD4 (Willey *et al.*, 1992 *b*). While we cannot rule out involvement of a heretofore unreported cellular protease inhibited by LC, the simplest interpretation is that HIV Vpu/Env-induced degradation of CD4 is mediated by the proteasome. Since the proteasome inhibitors did not completely block CD4 degradation, we cannot rule out the possibility that CD4 is degraded by both proteasome and non-proteasome mechanisms. Proteasome-mediated degradation would have a parallel in the ability of HPV E6 to bind the tumour suppresser protein p53 and induce its rapid, proteasome-mediated degradation (Scheffner *et al.*, 1993) and the ability of the CMV US11 protein to induce rapid, proteasome-mediated degradation of the MHC class I heavy chain (Wiertz *et al.*, 1996). We also noted an intriguing amino acid sequence similarity between Vpu and sequences in IκB known to be crucial for proteasome-mediated degradation. Our speculation that HIV-1 may have transduced this cellular motif to provide part of a signal involved in proteasome-mediated degradation suggests a new way in which viruses may co-opt cellular machinery for their own purposes.

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