A Conserved Dileucine Motif Mediates Clathrin and AP-2-dependent Endocytosis of the HIV-1 Envelope Protein

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During the assembly of enveloped viruses viral and cellular components essential for infectious particles must colocalize at specific membrane locations. For the human and simian immunodeficiency viruses (HIV and SIV), sorting of the viral envelope proteins (Env) to assembly sites is directed by trafficking signals located in the cytoplasmic domain of the transmembrane protein gp41 (TM). A membrane proximal conserved GYxxØ motif mediates endocytosis through interaction with the clathrin adaptor AP-2. However, experiments with SIV_{mac239} Env indicate the presence of additional signals. Here we show that a conserved C-terminal dileucine in HIV_{HXB2} also mediates endocytosis. Biochemical and morphological assays demonstrate that the C-terminal dileucine motif mediates internalization as efficiently as the GYxxØ motif and that both must be removed to prevent Env internalization. RNAi experiments show that depletion of the clathrin adaptor AP-2 leads to increased plasma membrane expression of HIV Env and that this adaptor is required for efficient internalization mediated by both signals. The redundancy of conserved endocytosis signals and the role of the SIV_{mac239} Env GYxxØ motif in SIV pathogenesis, suggest that these motifs have functions in addition to endocytosis, possibly related to Env delivery to the site of viral assembly and/or incorporation into budding virions.

INTRODUCTION

The assembly of enveloped animal viruses requires that the viral and cellular components needed to make infectious particles are brought to the same site within the infected cell at the same time. For the primate lentiviruses (the human immunodeficiency viruses types 1 and 2 [HIV-1 and -2] and the simian immunodeficiency viruses [SIV]) the key viral structural proteins required to generate infectious particles are Gag, Gag-Pol, and Env. Although Gag alone can promote the formation of virus-like particles, Env and Gag-Pol are both essential for the formation of infectious virions. Although a considerable amount is known about these proteins, little is understood of the mechanisms through which they are targeted to the sites of assembly in infected cells.

In many cell types, HIV assembles at the plasma membrane and, in the course of Gag assembly, the particles derive their membrane from the plasma membrane. For these particles to be infectious, Env must be transported to the cell surface, but the level to which it is incorporated into budding virions is unclear. Recent data have suggested that fewer than 10 Env protein complexes (trimers) are incorporated per virion (Chertova *et al.*, 2002; Zhu *et al.*, 2003), and early studies of HIV infected T-cells showed that much of the newly synthesized Env is transported to lysosomes and

degraded (Willey *et al.*, 1988). However, it has recently become evident that in macrophages the assembly of Env-containing infectious HIV occurs on intracellular membranes, that have some characteristics in common with late endosomes (Raposo *et al.*, 2002; Pelchen-Matthews *et al.*, 2003). The assembly of virus in distinct locations suggests that Env must contain the necessary trafficking information to ensure that its transport is coordinated with that of Gag. This information, and its appropriate interpretation in the infected cell, is likely to be essential for productive infection and pathogenesis. Indeed, deletion of an Env membrane proximal sorting/endocytosis motif in SIV_{mac239} enhances the viral cytopathic effect in vitro and abrogates pathogenesis in vivo (Sauter *et al.*, 1996; Fultz *et al.*, 2001).

HIV and SIV Envs are type I integral membrane proteins that are made as 160-kDa precursor proteins on the endoplasmic reticulum, where they undergo trimerization and extensive glycosylation before being exported to the secretory pathway (Braakman and van Anken, 2000). During transport through the secretory pathway, or possibly within an endosomal compartment (Franzusoff *et al.*, 1995), the extracellular (luminal) domain of gp160 is proteolytically cleaved by furin or a furin-like protease to generate the surface unit (SU, gp120) and transmembrane (TM, gp41) proteins of the mature Env glycoprotein. The first ~650 amino acids (depending on the strain of virus) of gp160 form gp120 and the ectodomain of gp41, followed by a short transmembrane domain. The remaining amino acids, from approximately position 705 to the C-terminus, form the relatively long (~150 amino acids) cytoplasmic domain. This domain is essential for viral replication and pathogenesis in vivo although, for SIV at least, much of the cytoplasmic domain is unnecessary for growth in culture (Kodama *et al.*, 1989). The cytoplasmic domain is believed to play key roles

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Abbreviations used: Env, envelope glycoprotein; gp, glycoprotein; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SU, surface subunit of the viral envelope glycoprotein; TM, transmembrane subunit of the viral envelope glycoprotein.

in virus assembly and has been considered important for Env incorporation into virions and for Env interactions with the N-terminal matrix (MA) domain of HIV Gag (Cosson, 1996; Vincent *et al.*, 1999).

Previously, we analyzed the trafficking properties of SIV_{mac239} Env using chimeras containing the ectodomain and transmembrane domains of human CD4 fused to the cytoplasmic domain of Env, as well as native Env expressed from an alpha virus expression vector (LaBranche *et al.*, 1995; Sauter *et al.*, 1996; Bowers *et al.*, 2000). The CD4 chimeras offered the advantage that, while retaining similar trafficking properties to the native Env protein, they were more amenable to expression in stable cell lines, and as a consequence, to biochemical and morphological analysis (Sauter *et al.*, 1996; Bowers *et al.*, 2000). Using these approaches, we demonstrated that a conserved GYxxØ motif (where x is any amino acid and Ø is an amino acid with a large hydrophobic side chain) in the cytoplasmic domain of SIV Env can function as an endocytosis signal. The activity of this signal is dependent on a membrane proximal tyrosine (Y₇₂₃ in SIV_{mac239}) that is highly conserved in all HIV-1, HIV-2, and SIV isolates (LaBranche *et al.*, 1995). The corresponding tyrosine in HIV-1 Env (Y₇₁₂ in HIV-1_{HXB2}) also appears to function as an endocytosis signal (Rowell *et al.*, 1995; Ohno *et al.*, 1997; Boge *et al.*, 1998) and as a basolateral targeting motif in polarized cells (Lodge *et al.*, 1997). In addition to the GYxxØ motif, SIV Env contains at least one more endocytosis signal that remains to be mapped in detail (Bowers *et al.*, 2000). Previous studies had indicated that HIV and SIV Envs interact with clathrin adaptor complexes and that the membrane proximal GYxxØ motif can bind both AP-1 and AP-2 adaptors (Bowers *et al.*, 2000; Wyss *et al.*, 2001). In addition, a conserved dileucine motif in the C-terminus of HIV-1_{HXB2} Env TM can also bind the clathrin adaptor complexes, though the role of this interaction has remained obscure (Ohno *et al.*, 1997; Boge *et al.*, 1998; Berlioz-Torrent *et al.*, 1999; Wyss *et al.*, 2001).

Using HIV-1_{HXB2} Env, and CD4-HXB2 Env cytoplasmic domain chimeras, we now show that the C-terminal dileucine motif also mediates endocytosis, that the activity of this motif is functionally equivalent to that of the membrane proximal GYxxØ motif, and that the two signals operate independently and their activities are not additive. In the presence of either motif Env accumulates in intracellular organelles and only remains on the cell surface when both motifs are functionally defective. RNAi knockdown experiments indicate that both motifs operate through interaction with AP-2 and the clathrin-mediated endocytic pathway. This functional redundancy in endocytosis signals suggests that internalization of cell surface Env is important for viral pathogenesis. Moreover, as a functional GYxxØ motif is required for SIV pathogenesis (Fultz *et al.*, 2001), the membrane proximal motif may have roles other than endocytosis.

MATERIALS AND METHODS

Tissue Culture Reagents, Chemicals, Antibodies, and Cells

Tissue culture reagents and plastics were from Invitrogen (Paisley, United Kingdom), and chemicals were from Sigma-Aldrich Company (Poole, United Kingdom), unless otherwise indicated. The murine mAb against human CD4, Q4120 (IgG₁) (Healey *et al.*, 1990) was obtained from Dr. Q. Sattentau through the Centralised Facility for AIDS Reagents at the National Institute for Biological Standards and Control (Potters Bar, United Kingdom). The recombinant human mAb 2G12 (IgG₁) against HIV gp120 (Buchacher *et al.*, 1994) was obtained from Dr. H. Katinger through the same program. MAbs against clathrin heavy chain (#23, IgG₁) and the μ 2 subunit of AP-2 (AP50, IgG₁) were purchased from BD Transduction Laboratories (Mannheim, Germany), and the mAb against γ -adaptin (100/3, IgG_{2b}) was from Sigma-Aldrich. Human transferrin coupled to Alexa Fluor 594 (⁵⁹⁴Tf) was purchased from Molecular

Probes Invitrogen (Paisley, United Kingdom). Alexa Fluor 488- and 594-conjugated goat anti-mouse IgG and isotype specific anti-IgG₁ and IgG_{2b} antibodies were from Molecular Probes Invitrogen. Goat anti-human IgG-FITC was purchased from Perbio Science United Kingdom (Cheshire, United Kingdom).

Q4120 was radioiodinated using ¹²⁵I-labeled 3-(p-hydroxyphenyl)-propionic acid N-hydroxy-succinimide ester (Bolton and Hunter reagent) essentially as described (Pelchen-Matthews *et al.*, 1998). Specific activity was ~500 Ci/mmol. The radioiodinated protein was stored in 100- μ l aliquots at -20°C and was stable for several months.

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal calf serum (FCS; Biowest, Nuaille, France), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. For stable cell lines, expressing CD4-HIV Env constructs, the medium was supplemented with 750 μ g/ml G418.

Plasmids and Transfection

Chimeric proteins containing the CD4 ectodomain and membrane-spanning domains linked to the cytoplasmic domain of HXB2 Env (see Figure 2) were made using a unique HindIII restriction site engineered into the pT4b molecular clone of CD4 (provided by D. Littman, New York University, New York) at nt 1351 such that four residues from the CD4 cytoplasmic domain (-RCRH) were included in the constructs, as previously described (Sauter *et al.*, 1996; Bowers *et al.*, 2000). These residues act as a spacer to place the HIV Env membrane proximal endocytosis motif at a requisite distance from the membrane for functional activity (Jing *et al.*, 1990). Cloning of the CD4 fragment into pSP65 has been described (Sauter *et al.*, 1996). Restriction sites for HindIII and EcoRI were introduced into the HIV-1_{HXB2} Env cytoplasmic domain by PCR with the forward primer 5'-ATAGTGAATAAGCTTAG-CAGGGGATATTCACCATTA-3' and the reverse primer 5'-GCCGAATTCT-TATAGCAAATTCCTTTCCCAAGCCCTGCTTATTC-3'. The fragment was digested with HindIII and EcoRI and cloned into pSP64 (Promega Biotech, Southampton, United Kingdom). The CD4-containing sp65 and HIV Env cytoplasmic domain-containing sp64 plasmids were then digested with PvuI and HindIII and ligated to create an EcoRI fragment encoding CD4/HIV Env chimeric proteins. Site-specific mutagenesis using the Quickchange system (Stratagene, Amsterdam, The Netherlands) was used to convert the HindIII site to the original sequence using the forward primer 5'-AGGTGCCGGCAC-CGAAGAGTATAGGCAGGGATATTCACC-3' and the reverse primer 5'-GGTGAATATCCCTGCCTAACTCTTCGGTGCCGGCACCT-3' according to the manufacturer's instructions. The constructs were cloned into the expression vector pCR3.1 (Invitrogen) using a KpnI restriction site, introduced by PCR, upstream of the CD4 gene and the EcoRI site 3' of the HIV Env sequence. Mutations of potential trafficking motifs (see Figure 2) were introduced using the Quickchange system. Short tail constructs were generated by mutating the Gly codon at the position equivalent to 726 in HXB2 to a stop codon using the same method. All constructs were verified by sequencing and proteins with an appropriate molecular mass were identified by Western blotting for the CD4 domain.

Plasmids containing the genes encoding the CD4/HIV Env chimeras were transfected into HeLa cells using FuGENE (Roche, Lewes, United Kingdom) according to the manufacturer's instructions. Stable transfectants were selected using G418 (750 μ g/ml). Selected colonies were expanded and screened for expression by immunofluorescence using anti-CD4 antibodies.

A pSVIII plasmid encoding HIV-1_{HXB2} gp160 was provided by Dr. Robin Weiss (Wohli Virion Center, Windeyer Institute, UCL, London) and has been described (Helseth *et al.*, 1990). Mutations of potential trafficking motifs (see Figure 2) were introduced using Quickchange mutagenesis. Env expression plasmids were transfected into HeLa cells using FuGENE according to the manufacturer's instructions and expressed for 48 h before analysis. Sixteen hours before analysis the transfected cells were treated with 5 mM sodium butyrate to enhance Env expression.

Endocytosis Assays

Quantitative endocytosis assays were performed essentially as described (Pelchen-Matthews *et al.*, 1991). Briefly, $1.2\text{--}1.8 \times 10^5$ CD4/Env expressing cells were seeded in 16-mm-diameter wells in 24-well plates and grown to confluency over 2 d. The cells were cooled on ice, washed with BM (RPMI-1640 without bicarbonate, containing 0.2% bovine serum albumin [BSA] and 10 mM HEPES, pH 7.4), and incubated with 250 μ l BM containing 0.5 nM ¹²⁵I-Q4120 for 2 h on ice. Free antibody was then washed away, and samples set aside to determine the cell surface levels of CD4/Env. The remaining cells were then warmed to 37°C by immersion of the plates into 37°C BM. At the indicated times the plates were transferred to 4°C BM. For each time point at least four wells were used. For half of the wells, the cells were washed in 4°C PBS and harvested directly in 400 μ l 0.2 M NaOH and transferred to tubes for γ -counting to determine the total cell-associated radioactivity. To determine the intracellular activity, the remaining cells were rinsed twice with 0.5 ml 4°C BM adjusted to pH 2.3 and then incubated twice for 3 min with the same medium to remove cell surface-bound antibody. These cells were then harvested in NaOH as above. The proportion of the internalized activity for each time point was determined by dividing the acid-resistant activity by the total

cell-associated activity, and the endocytosis rates were calculated by analysis of data from the first 5 min of warm-up as described (Bowers *et al.*, 2000).

RNAi Knockdown

Small interfering RNAs (siRNA) against the $\mu 1$ subunit of AP-1 (Hirst *et al.*, 2003) and the $\mu 2$ subunit of AP-2 (Fraile-Ramos *et al.*, 2003) have been described and were purchased from Xeragon (Zurich, Switzerland). Cells were transfected with siRNA by nucleofection (Amaxa, Koeln, Germany) using the nucleofection program A-28 for HeLa cells. Typically a 70–80% confluent 10-cm dish of cells was used for up to four nucleofection reactions. For $\mu 1$ knockdown, the cells were transfected with 300 pmol of siRNA, and the entire reaction was plated onto one 10-cm dish and incubated for 3 d at 37°C. The cells were then nucleofected again with 150 pmol siRNA and incubated for a further 3 d before analysis (Lui-Roberts *et al.*, 2005). For $\mu 2$ knockdown, cells were nucleofected once with 150 pmol siRNA, plated directly onto coverslips, and incubated for 56 h before analysis by antibody- and transferrin-uptake experiments.

Antibody and Transferrin Uptake

For antibody feeding, HeLa cells expressing CD4/HIV Env constructs or HxB2 Env were grown on coverslips for 2 d and then washed twice in BM and incubated for 15 min to 2 h, as indicated, in 37°C BM containing 4.5 $\mu\text{g}/\text{ml}$ anti-CD4 (Q4120) or 20 $\mu\text{g}/\text{ml}$ anti-gp120 (2G12). The cells were then cooled on ice, washed twice in 4°C BM to remove unbound antibody, fixed in 2.5% formaldehyde, and processed for immunofluorescence microscopy.

For transferrin (Tf) feeding experiments, $\mu 2$ siRNA-transfected HeLa cells were washed with BM and incubated for 30 min at 37°C to remove endogenous Tf. The cells were then incubated in BM containing 200 nM Tf coupled to Alexa⁵⁹⁴ (Molecular Probes, Invitrogen) at 37°C. After 10 min the coverslips were placed on ice and washed with cold PBS. The cells were then fixed and processed for immunofluorescence microscopy. Alternatively, ⁵⁹⁴Tf was added for the last 10 min of antibody feeding.

Immunofluorescence

Cells on glass coverslips were fixed for 20 min with 2.5% formaldehyde in serum-free medium supplemented with 20 mM HEPES (pH 7.0) at room temperature. The cells were then washed twice with serum-free medium containing 20 mM HEPES, followed by incubation in phosphate-buffered saline containing 1 mM Mg^{2+} and 0.5 mM Ca^{2+} (PBS^{2+}) at 4°C for 10 min. Subsequently, the cells were permeabilized and blocked in PBS^{2+} containing 20% fetal calf serum, 15 mM glycine, 20 mM HEPES, and 0.05% saponin for 15 min at room temperature. For surface staining, the same buffer without saponin was used. All subsequent incubations and washing steps were carried out in the permeabilization/blocking buffer. The cells were incubated for 45 min at room temperature with antibodies against CD4 or gp120. After extensive washing, the cells were incubated at room temperature in the dark for 30 min with Alexa-labeled secondary antibodies. The coverslips were then washed five times in permeabilization buffer, twice in PBS^{2+} , and quickly in water before mounting on glass slides with Mowiol. The cells were examined at ambient temperature through a 60 $\times$ oil immersion lens (NA 1.4) on a Nikon Optiphot 2 microscope (Kingston upon Thames, United Kingdom) fitted with a MRC 1024 confocal laser scanner (Bio-Rad Laboratories, Hemel Hempstead, Herts, United Kingdom). Images were acquired using the Bio-Rad Lasersharp software and imported into Adobe Photoshop CS2 and Illustrator CS2 (San Jose, CA) to generate figures.

Western Blotting

Mock and $\mu 2$ siRNA transfected cells were cultured in six-well plates, and the cells were scraped into 50 μl lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, complete protease inhibitor cocktail [Roche, Lewes, United Kingdom]) and lysed for 30 min on ice. Cellular debris were removed by centrifugation at 13,200 rpm for 15 min in a bench top microfuge, and the supernatants were diluted 3:1 in 4 $\times$ nonreducing SDS-PAGE sample buffer. The samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose, and analyzed essentially as described (Fraile-Ramos *et al.*, 2001).

RESULTS

Cellular Distribution of HIV Env and Env Cytoplasmic Domain Mutants

The conserved GY₇₁₂xxØ motif in the cytoplasmic domain of HIV Env was previously shown to mediate Env endocytosis (Rowell *et al.*, 1995; Ohno *et al.*, 1997; Boge *et al.*, 1998; Wyss *et al.*, 2001). However, mutation of Y₇₁₂ did not abrogate internalization, indicating that additional motifs C terminal to Y₇₁₂ must be also capable of mediating endocytosis (Rowell *et al.*, 1995). Moreover, in *in vitro* assays, the GY₇₁₂xxØ motif

as well as two dileucine motifs at positions 814/815 and 855/856 in the cytoplasmic domain can bind the $\mu 1$ subunit of the clathrin adaptor AP-1 (Ohno *et al.*, 1997; Boge *et al.*, 1998; Berlioz-Torrent *et al.*, 1999; Wyss *et al.*, 2001).

To characterize the signals mediating Env trafficking and endocytosis, we analyzed the distribution of HxB2 Env (referred to as construct “Y wt”) in HeLa cells by immunofluorescence 48 h after transfection. We stained permeabilized cells with a human mAb that recognizes a carbohydrate epitope on gp120 (2G12) and reacts specifically with post-ER forms of the protein (Buchacher *et al.*, 1994). We found that little Env was expressed at the plasma membrane and that the bulk of the protein localized to the perinuclear area of the cell in a vesicular pattern (Figure 1A, top left panel). To further analyze Env distribution, we performed antibody uptake assays. HeLa cells transiently expressing Env constructs were incubated with 2G12 for 2 h at 37°C and then fixed and labeled, intact or after permeabilization, with a secondary FITC-coupled anti-human antibody. This protocol allowed us to examine exclusively the Env population that had reached the plasma membrane and either remained at this site or undergone endocytosis. Little labeling was seen on intact cells (not shown), but on permeabilized cells a labeling pattern similar to that observed above was seen (top left panel in Figure 1B). These observations indicated that the steady state distribution of Env was achieved, at least in part, by transport of the protein to the plasma membrane and subsequent endocytosis.

To analyze the role of the potential trafficking motifs in the surface expression and intracellular distribution of Env, we generated a set of mutations in HxB2 Env as indicated in Figure 2. We disrupted the GYxxØ motif by changing Y₇₁₂ to I (Y₇₁₂I) and investigated the role of the dileucine motifs at positions 814/815 and 855/856 by mutations to dialanine (Y + LL_{814/815}AA and Y + LL_{855/856}AA). The mutants were transiently transfected into HeLa cells and their distribution was analyzed by immunofluorescence confocal microscopy. Mutation of Y₇₁₂ to I, which inactivates the GYxxØ motif as a sorting signal, did not markedly change the distribution of Env compared with the wt. The bulk of the protein was still found in the perinuclear area of expressing cells, suggesting that the mutant retains the ability to undergo endocytosis though the distribution was a little more disperse (Figure 1A). When the dileucine motifs were changed to AA, Env localization was almost identical to the wt Env, although the distribution of the Y + LL_{855/856}AA construct was similar to the Y₇₁₂I mutant. Constructs containing a combination of LL_{814/815}AA and Y₇₁₂I mutations (I + LL_{814/815}AA) had a similar distribution to Y₇₁₂I. However, combination of LL_{855/856}AA with Y₇₁₂I (I + LL_{855/856}AA) produced a protein that was redistributed to the plasma membrane with little evidence of intracellular protein (Figure 1A).

This result, suggesting that the C-terminal dileucine might play a role in Env internalization, was supported by antibody uptake experiments (Figure 1B). After 2 h feeding, the distribution of the anti-Env Ab was in all cases similar to the steady state distribution. Intracellular, perinuclear labeling was seen for all constructs except for the double I + LL_{855/856}AA mutant, where the plasma membrane labeling was even more pronounced than in the steady state labeling. We conclude that, as with SIV Env, HxB2 Env contains at least two signals that mediate endocytosis and that, in addition to GY₇₁₂xxØ, LL_{855/856} but not LL_{814/815} acts as an endocytosis motif.

Figure 1. Cellular localization of HIV Env mutants. (A) HeLa cells transiently expressing HxB2 Env or the indicated HxB2 mutants, were fixed, permeabilized, and stained with a human anti-Env mAb (2G12) followed by a mouse anti-human antibody coupled to FITC. The steady state distribution of Env 48 h after transfection is shown. (B) The ability of the proteins to reach the cell surface was assessed by incubating live, Env-expressing cells in media containing 20 μ g/ml 2G12 for 2 h at 37°C before fixation, permeabilization, and staining with anti-human FITC. The staining in these cells indicates Env that had been exposed at the plasma membrane during the 2-h incubation. Env negative cells fail to show labeling with 2G12 with either protocol (data not shown). All images show single 0.5- μ m confocal sections. Scale bar, 10 μ m.

The C-terminal Dileucine Acts as an Endocytosis Signal

To compare the functional activities of the GYxxØ and LL_{855/856} motifs as endocytosis signals, we determined the

internalization rates of proteins carrying either one or both of the putative signals. Because gp160 constructs are not expressed efficiently in stable cell lines, reproducible bio-

Figure 2. CD4-HIV Env chimeras and HxB2 Env cytoplasmic domain mutants. The amino acid sequence of the HxB2 (HIV-1 Subtype B) gp41 cytoplasmic domain is shown with potential trafficking motifs shaded. Amino acids are numbered as described (Korber *et al.*, 1998). CD4-Env chimeras were created by fusion of the gp41 cytoplasmic domain (gp41 cyt) to the ectodomain and transmembrane domains of CD4 as described in *Materials and Methods*. Mutations were introduced as indicated. Short tail constructs were created by mutation of the codon for amino acid 726 to a stop codon. HxB2 Env constructs were generated by mutating the sequence of gp160 in vector pSVIII as described in *Materials and Methods*. Constructs combining Y₇₁₂I mutations with dileucine mutations are named I + LL_{814/815}AA and I + LL_{855/856}AA, respectively.

Figure 3. Localization of CD4-HIV Env chimeras. HeLa cell lines stably expressing CD4-Env chimera were fixed, permeabilized, and stained for CD4 with Q4120 and anti-mouse Alexa 488 to visualize steady state expression (A) or fed with Q4120 for 2 h at 37°C, and then fixed, permeabilized, and stained with anti-mouse Alexa 488 to show the CD4-HIV Env pool that has trafficked over the plasma membrane (B). All panels are single confocal sections. Scale bars, 10 μ m.

chemical assays with native Env are not currently feasible. We therefore chose to use chimeric proteins, where the cytoplasmic domain of HxB2 gp41 was fused to the luminal and membrane spanning domains of CD4, as previously described for SIV Env (Sauter *et al.*, 1996; Bowers *et al.*, 2000). The set of constructs used is shown in Figure 2. We also included two short tail variants, where a stop codon was introduced to replace the G₇₂₆ codon. Thus, construct "CD4-Y short" contained only the membrane proximal GYxxØ signal, and "CD4-I short" was not expected to contain any endocytosis information, similar to SIV_{CP-mac} Env that contains a Tyr to Cys mutation on the background of a truncated cytoplasmic tail (LaBranche *et al.*, 1994). These constructs were used to generate stable HeLa cell lines.

To confirm that the CD4-Env chimeras are a reliable model for native Env, the subcellular localization of the chimeras was analyzed by immunofluorescence (Figure 3). As expected, staining for a chimera containing the native HxB2 Env tail (CD4-Y wt) showed almost no surface labeling and localized to the perinuclear area of the transfected cells. This was also the case for CD4-Y_{712I}, CD4-Y + LL_{814/815}AA, and CD4-Y + LL_{855/856}AA (Figure 3A). CD4-Y short localized predominantly to internal vesicles, but these were more dispersed than for CD4-Ywt, suggesting that the cytoplasmic domain may contain information crucial for correct intracellular trafficking. Mutation of LL_{814/815}AA in combination with Y_{712I} (CD4-I + LL_{814/815}AA) also generated a protein that was predominantly intracellular, though the distribution of this chimera appeared to be more dispersed and present at the plasma membrane to a greater

extent than the corresponding Env variant. The CD4-I short and CD4-I + LL_{855/856}AA constructs were localized predominantly at the plasma membrane as expected, because these mutants should lack both of the putative endocytosis motifs (Figure 3A).

To support the morphological analysis, we quantified the cell surface expression of the different CD4-Env chimeras using radiolabeled antibody Q4120 against CD4. The data of a representative experiment is shown in Figure 4A, where the amount of radioactivity bound to the surface of cells expressing the different constructs is displayed as a multiple of the radioactivity bound to cells expressing CD4-Ywt. Consistently, the binding of antibody to the CD4-Ywt cells was low. However, mutation of Y_{712I}, in combination with truncation of the cytoplasmic domain or the mutation of LL_{855/856}AA, increased the surface expression 6–12-fold in agreement with the morphological data illustrated in Figure 3.

Plasma membrane targeting and internalization of the CD4-Env chimeras was assessed in antibody uptake assays, essentially as described above, using Q4120. As for the Env constructs, the CD4-Env chimeras showed intracellular staining, consistent with the idea that the proteins reached the cell surface and were subsequently internalized (compare Figures 1B and 3B). The low level of antibody labeling at the cell surface suggested this internalization was very efficient. All constructs showed evidence of internalization, with the exception of the variants CD4-I short and CD4-I + LL_{855/856}AA, where labeling primarily at the plasma membrane showed that endocytosis of these constructs was reduced or abolished (Figure 3B). Together, these observations

Figure 4. Surface expression and endocytosis of CD4-HIV Env chimeras. HeLa cells expressing the CD4-HIV Env chimera were incubated with ^{125}I -Q4120 for 2 h at 4°C. The free antibody was washed away and the bound radioactivity measured. The amount of labeled antibody bound to CD4-HIV Env mutants was plotted as a multiple of the amount bound to CD4-Ywt (A). Alternatively, endocytosis was initiated by immersing the plates in BM at 37°C. At the end of the indicated time periods, the cells were transferred to ice-cold BM and washed, and the total and intracellular radioactivity was determined as described in *Materials and Methods*. The graphs show the intracellular (acid-resistant) radioactivity as a percentage of the total cell-associated radioactivity at each time point for representative cell lines (B). The error bars indicate the SEs from the mean calculated for several assays for each cell line. The data for all assays is summarized in Table 1.

indicated that the CD4-Env chimeras are a good model for HIV Env trafficking and provided further evidence for a role for the C-terminal dileucine motif in Env endocytosis.

To measure the endocytosis activity of the different constructs, we used ^{125}I -Q4120 in endocytosis assays, as previously described (Pelchen-Matthews *et al.*, 1991). Representative endocytosis curves for all constructs are shown in Figure 4B, and the results of several experiments are summarized in Table 1. Chimeras containing the complete HIV Env cytoplasmic domain (CD4-Ywt) were internalized rapidly ($\sim 6\%/min$ in the first 5 min after warming to 37°C) and to a high extent (80% of the total after 60 min; Figure 4B and Table 1). A similar endocytosis rate was also seen for CD4-Y short, confirming that the GYxxØ signal can mediate efficient internalization as previously shown (Boge *et al.*, 1998; Wyss *et al.*, 2001). However, mutation of Y₇₁₂ to I did not affect the rates of uptake of constructs with a full-length cytoplasmic domain, indicating the presence of a second signal capable of mediating efficient internalization. By contrast, the CD4-I short construct was internalized slowly (approximately 0.8%/min; Table 1 and Figure 4B) and to low

levels (10% of the total after 60 min) comparable to the bulk flow endocytosis of CD4 molecules lacking a cytoplasmic

Table 1. Endocytosis of CD4-spacer-HIV Env chimeras in stable HeLa lines

Chimera type	Endocytosis rate (%/min) ^a	Intracellular (%) ^b
Ywt	6.0 ± 1.2	81 (6)
Y712I	6.7 ± 1.9	86 (4)
Y short	6.5 ± 3.6	92 (4)
I short	0.8 ± 0.5	10 (3)
Y + LL814/815AA	5.1 ± 1.4	77 (2)
Y + LL855/856AA	6.0 ± 1.6	78 (5)
I + LL814/815AA	4.3 ± 0.1	64 (2)
I + LL855/856AA	0.3 ± 0.1	15 (3)

^a Endocytosis rates were calculated for the first 5 min after warming to 37°C as the mean ± SD.

^b Proportion of initial cell surface counts that are intracellular after 60 min at 37°C, with the indicated number of assays in parentheses.

Figure 5. AP-2 is required for normal CD4-Env distribution. HeLa cells expressing CD4-HIV Env constructs were transfected with a siRNA against the $\mu 2$ subunit or with buffer only (mock). After 56 h the cells were analyzed for $\mu 2$ expression by Western blot (A) using clathrin heavy chain as a loading control. At the same time cells were fed with Alexa-Fluor 594-conjugated Tf for 10 min at 37°C before fixation. Cells were then permeabilized and stained with Q4120 to show steady state distribution of CD4-Env by single confocal sections (B). Intact cells were stained with Q4120 and analyzed for cell surface expression of CD4-Env (C), projections of Z-series stacks of confocal sections are shown. In both B and C a redistribution of CD4-Env constructs to the cell surface was observed. Scale bars, 10 μ m.

domain (Pelchen-Matthews *et al.*, 1992; Pitcher *et al.*, 1999). These results were consistent with the high surface expression of this construct (Figures 3 and 4A). For LL_{814/815}AA mutants, with either Y₇₁₂ or Y₇₁₂I, the rates and extents of internalization were reduced only moderately (i.e., ~20%) compared with CD4-Y wt, indicating that the CD4-I + LL_{814/815}AA construct retained a functional endocytosis motif. By contrast, the CD4-I + LL_{855/856}AA construct showed a reduced internalization rate similar to that found for CD4-I short, indicating that the functional endocytosis motifs had been removed.

Together these data indicate that the cytoplasmic domain of HxB2 Env contains two functionally redundant endocytosis motifs: the membrane proximal GY₇₁₂xxØ motif and the C-terminal dileucine motif (LL_{855/856}). Both motifs act with equivalent efficacy and show no significant additive effect. Little if any endocytic trafficking activity is associated with the dileucine motif at 814/815.

Endocytosis of CD4/HIV Env Chimeras Is Dependent on the Clathrin Adaptor AP-2

It was previously shown that HIV and SIV Env cytoplasmic domains can bind the clathrin AP-1 and AP-2 adaptor complexes through the conserved GYxxØ motif (Ohno *et al.*, 1997; Boge *et al.*, 1998; Berlioz-Torrent *et al.*, 1999; Bowers *et al.*, 2000; Wyss *et al.*, 2001). Pulldown assays have also suggested that LL_{814/815} and LL_{855/856} bind AP-1 (Wyss *et al.*, 2001), but no previous evidence has suggested that either of these motifs bind AP-2 or that the C terminal LL motif can mediate endocytosis of HIV Env. To examine the functional activities of these signals in more detail, we used siRNA against the $\mu 2$ subunit of AP-2, which have been demonstrated to inhibit AP-2-mediated endocytosis (Fraile-Ramos *et al.*, 2003; Motley *et al.*, 2003). HeLa cells expressing CD4-Env constructs were treated with the siRNA, and the levels of $\mu 2$ expression were analyzed 56 h after nucleofection by Western blot. To check protein loading, blots were also

Figure 6. AP-2 is required for CD4-Env internalization. HeLa cells expressing CD4-Env constructs were transfected with an siRNA to reduce expression of the $\mu 2$ subunit of the AP-2 complex or buffer only (mock). After 56 h the cells were fed with Q4120 anti-CD4 for 15 min at 37°C (A) or for different time points as indicated (B). Alexa-Fluor 594-conjugated Tf was added for the last 10 min of feeding. Cells were then fixed, permeabilized, and stained with an Alexa-Fluor 488 coupled secondary anti-mouse antibody. Projections of confocal Z-series are shown in A and B. The small panels in A represent the bottom (b) and middle (m) sections of the Z-series stack for CD4 staining shown on the left. Scale bars, 10 μ m.

probed with an anti-clathrin heavy-chain antibody. Typically siRNA reduced the expression of $\mu 2$ by 80% (Figure 5A). To test whether loss of $\mu 2$ affected endocytosis, siRNA and mock-treated cells were incubated with Alexa 594-coupled human diferric transferrin (594 Tf) for 10 min at 37°C, and the distribution of 594 Tf was assessed by fluorescence microscopy. In mock-treated cells, Tf was seen within endocytic vesicles, with little labeling at the cell surface (Figure 5B). By contrast, the majority of $\mu 2$ siRNA-treated cells showed Tf accumulation at the plasma membrane, suggesting that endocytosis was inhibited. A minor fraction of

siRNA treated cells did internalize Tf, suggesting that these cells had not taken up the siRNA or that AP-2 knockdown was incomplete (Figures 5, B and C, and 6A).

To examine how $\mu 2$ siRNA affected trafficking of the CD4-Env constructs, siRNA transfected and 594 Tf-fed, CD4-Env-expressing HeLa cells were stained with anti-CD4 antibody. We observed intracellular CD4-Y wt protein in both mock- and siRNA-treated cells. However, plasma membrane expression was increased in the $\mu 2$ knockdown cells, indicating that CD4-Y wt internalization from the plasma membrane was reduced or even stopped (Figure 5B). We

assume that the intracellular pool of CD4-Y wt represented protein that had not been cycling via the plasma membrane. The same change in distribution was observed for the CD4-Y + LL_{855/856}AA mutant, where endocytosis is mediated entirely by the GYxxØ motif. To assess whether μ 2 knockdown also affects endocytosis mediated by the C-terminal dileucine motif, we analyzed the distribution of CD4-Y₇₁₂I constructs and found that plasma membrane expression was increased in the siRNA transfected cells (Figure 5B). To further assess the observed redistribution to the plasma membrane and visualize the surface fraction of the protein, we stained intact siRNA- and ⁵⁹⁴Tf-treated cells with anti-CD4 antibody. It was apparent that mock-treated cells for all three CD4-Env constructs had very low plasma membrane expression of CD4-Env (Figure 5C). However, RNAi treatment led to an increase in plasma membrane expression levels, confirming the redistribution of CD4-Env in AP-2 knockdown cells.

Analysis of the steady state distribution could not determine whether the increased plasma membrane expression was indeed due to a reduction in endocytosis or resulted from an increase in transport to the plasma membrane. We therefore performed antibody uptake experiments, incubating mock- and μ 2 siRNA-transfected CD4-Env-expressing HeLa cells with anti-CD4 for 15 min and adding ⁵⁹⁴Tf for the last 10 min of incubation. In mock-treated cells the majority of the antibody labeled CD4-Env was internalized within the 15-min incubation (Figure 6A) and gave a distribution similar to that seen for the steady state staining (Figure 5B). Efficient internalization was also observed for the construct with the wt cytoplasmic domain as well as for the two mutants lacking one of the two internalization motifs. μ 2 siRNA treatment led to accumulation of CD4-Env at the plasma membrane, indicating that AP-2 is required for efficient endocytosis (Figure 6A). This effect was observed for all three constructs, providing evidence for a role of AP-2 in Env internalization mediated by either the GYxxØ or the C-terminal dileucine motif. However, when antibody feeding was extended to 30 min or longer, internal pools of CD4-Env were also seen in the siRNA-treated cells (Figure 6B). Whether this uptake was due to some remaining AP-2 activity or reflects either AP-2-independent clathrin-mediated endocytosis (Motley *et al.*, 2003) or a clathrin-independent mechanism for Env internalization is unclear.

To analyze whether the previously described interaction between HIV Env and the AP-1 clathrin adaptor and the observed recruitment of AP-1 by HIV Env to the TGN (Wyss *et al.*, 2001) are directly relevant to HIV Env distribution and to examine a role for AP-1 in Env endocytosis, we used siRNA against the μ 1 subunit of AP-1 (Hirst *et al.*, 2003). Because the incomplete AP-1 complexes in μ 1-deficient cells cannot be recruited onto membranes (Meyer *et al.*, 2000) and the remaining subunits are destabilized, the γ -subunit of AP-1 became cytosolic in μ 1 RNAi treated cells (Figures 7A and 7B; Lui-Roberts *et al.*, 2005), compared with its normal Golgi-associated, perinuclear/vesicular distribution in mock-treated cells. The impact of μ 1 depletion on CD4 Env distribution was again assessed by steady state staining (Figure 7A) and antibody feeding (Figure 7B). We found that in both cases CD4 Y wt distribution was similar in mock- and in RNAi-treated cells, with the CD4-Env protein found predominantly in perinuclear vesicles. Thus, in μ 1-depleted cells, the CD4-Env protein traffics to the plasma membrane and is then internalized, suggesting that AP-1 is not required for CD4-Env endocytosis and is not essential for its transport to the plasma membrane.

Figure 7. Depletion of the μ 1 subunit of the AP-1 complex by RNAi. HeLa cells expressing CD4-Y wt were transfected with a siRNA against the μ 1 subunit. After 3 d the cells were transfected again with the same siRNA. Q4120 staining of fixed cells (A) or Q4120 uptake assays (B) were performed 3 d after the second transfection. All cells were labeled for γ -adaptin. μ 1 knockdown is indicated by a diffuse γ -adaptin distribution. CD4-HIV Env distribution did not appear to be affected in either case by the knockdown. Scale bars, 10 μ m.

Endocytosis of HxB2 Env Is Dependent on the Clathrin Adaptor AP-2

To analyze whether the endocytosis of the full-length HxB2 is also dependent on AP-2, we performed RNAi experiments in combination with HxB2 Env transfection. HeLa cells treated with μ 2 siRNA as described above were supertransfected 12 h later with HxB2 Env constructs and subsequently analyzed by 2G12 feeding for 15 min. To control for μ 2 knockdown, ⁵⁹⁴Tf was added during the last 10 min of the antibody incubation. Subsequent staining of fixed cells with FITC-coupled anti-human secondary reagent revealed that in mock-treated cells Env was internalized to intracellular vesicles within 15 min (Figure 8). Internalization was detected for Y wt and the mutant constructs Y₇₁₂I and Y + LL₈₅₆AA lacking one of the two internalization motifs. Similar to the CD4-Env chimera, μ 2 siRNA treatment led to the accumulation of HxB2 Env at the plasma membrane (Figure 8). Again, this effect was observed for all three constructs, supporting the experiments with the CD4-Env chimera (Fig-

Figure 8. AP-2 is required for Env internalization. HeLa cells were nucleofected with an siRNA to reduce expression of the $\mu 2$ subunit of the AP-2 complex or with buffer alone (mock). After 12 h the cells were transfected with HxB2 Env mutants as indicated. The cells were fed 48 h later with 2G12 anti-gp120 for 15 min at 37°C. Alexa-Fluor 594-conjugated Tf was added for the last 10 min of feeding. Cells were then fixed, permeabilized, and stained with a FITC-coupled secondary anti-human antibody. Single confocal sections are shown. Scale bars, 10 μ m.

ure 6) and confirming that the AP-2 adaptor complex is important for HIV Env internalization mediated by either the GYxxØ or the C-terminal dileucine motif.

DISCUSSION

The envelope glycoproteins of primate immunodeficiency viruses are crucial components of viral particles being essential for efficient viral infectivity by binding the viral receptor/coreceptor on host cells and mediating membrane fusion. In addition, as an external component of viral particles, they are a primary target for host antibody responses and are of major interest for vaccine development (Wyatt and Sodroski, 1998). As an enveloped virus HIV assembles at a membrane interface where oligomerization of Gag complexes is coordinated with the incorporation of Env into budding particles. Thus, it is essential that Env is targeted to virus assembly and budding sites in the cell. However, the precise site of assembly varies in different HIV target cells. In T-cells assembly occurs predominantly at the plasma membrane, whereas in macrophages HIV assembles on intracellular membrane-bound multivesicular organelles (Raposo *et al.*, 2002; Pelchen-Matthews *et al.*, 2003). Thus Env and Gag must contain appropriate trafficking signals to allow infectious particle assembly in distinct locations. Although some knowledge of intracellular Gag trafficking has been gained (Perlman and Resh, 2006; Ryzhova *et al.*, 2006), little is known about Env targeting in infected cells.

Previously, several labs have identified trafficking signals in HIV and SIV Env that mediate endocytosis (LaBranche *et al.*, 1995; Rowell *et al.*, 1995; Bowers *et al.*, 2000; Wyss *et al.*, 2001), polarized sorting (Lodge *et al.*, 1997), and recycling to the TGN (Blot *et al.*, 2003), through interactions with diverse components of the endocytic trafficking machinery including the clathrin-adaptors AP-1 and AP-2 and the putative late endosome-Golgi recycling protein TIP47 (Blot *et al.*, 2003). Of these motifs, the highly conserved membrane proximal GYxxØ motif is the most extensively characterized. This motif binds both AP-1 (Boge *et al.*, 1998; Bowers *et al.*, 2000) and AP-2 adaptors (Ohno *et al.*, 1997; Boge *et al.*, 1998; Berlioz-Torrent *et al.*, 1999; Wyss *et al.*, 2001) and mediates endocytosis from the cell surface (LaBranche *et al.*, 1995; Rowell *et al.*, 1995; Bowers *et al.*, 2000; Wyss *et al.*, 2001). Quantitative experiments with CD4-SIV Env constructs suggest that any Env delivered to the plasma membrane resides there for <5 min before removal by endocytosis (Bowers *et al.*, 2000). Moreover, for SIV_{mac239} at least, the GYxxØ motif is essential for pathogenesis (Fultz *et al.*, 2001).

Our previous experiments with SIV_{mac239} Env suggested that, in addition to the conserved GYxxØ signal, more endocytosis information is present in the full-length Env protein. Thus the internalization rate of a CD4-SIV Env chimera was reduced only 50% when the GYxxØ signal was eliminated (Bowers *et al.*, 2000). Here we show that the same is true for HIV_{HxB2} Env and identify the C-terminal dileucine as the relevant second signal. Dileucine motifs have previ-

ously been identified as endocytosis and trafficking signals in studies with cellular proteins, including MHC-II, CD4, and CI-M6PR, and are believed to interact with clathrin adaptors (Marks *et al.*, 1996), though the mode of binding is different from that of GYxxØ motifs (Bonifacino *et al.*, 1996). Indeed the dileucine motifs at positions 814/815 and 855/856 in HxB2 Env have previously been shown to bind AP-1 complexes. However, the roles of these motifs in HIV Env trafficking have remained obscure (Wyss *et al.*, 2001). Although our previous experiments suggested the C-terminal dileucine motif in SIV_{mac239} Env does not contribute to endocytic trafficking (Bowers *et al.*, 2000), we demonstrate here that the C-terminal dileucine in HxB2 Env clearly acts as an endocytosis signal operating through clathrin (data not shown) and the clathrin adaptor AP-2. This signal is independent of the GYxxØ motif, but is equally efficient, and both the C-terminal dileucine, and the GYxxØ motifs must be removed to allow efficient cell surface expression of Env. Such double mutants show only basal levels of endocytosis, indicating that the two motifs account for all the endocytosis information. In some cases, dileucine-based sorting signals require an acidic amino acid at position -4 and/or -5 relative to the first leucine (Letourneur and Klausner, 1992; Pond *et al.*, 1995). The C-terminal dileucine of HIV-1 has a glutamic acid at position -3 (aa 852 in HxB2). However, mutation of this residue did not change the distribution of Env in cells, nor did it reduce the endocytosis of the CD4-Y₇₁₂I mutant for which internalization is entirely governed by the dileucine motif (data not shown). By contrast, LL_{814/815} does not apparently contribute to endocytosis and its role in Env trafficking remains unclear. Potential involvement of LL_{814/815} in Env targeting to the viral assembly site is currently under investigation. Significantly, our results here suggest that there are differences in the trafficking signals in SIV and HIV Env, which may explain why short tail Env variants emerge during SIV propagation in cultured human T-cell lines, but similar HIV variants do not (Kodama *et al.*, 1989; Luciw *et al.*, 1998; Shacklett *et al.*, 2000).

How trafficking signals in Env are coordinated with Gag in relevant host cells remains to be established. In the main, HIV is thought to assemble at the plasma membrane of T-cells. The finding that endocytosis signals function to maintain low levels of Env on the cell surface may explain why HIV particles collected from cultured T-cell lines have relatively low Env levels (average 7–10 spikes/virion; Chertova *et al.*, 2002; Zhu *et al.*, 2003). In addition, the levels of Env expression on the cell surface and on virions could be a determinant for host humoral immune responses and viral evasion (Yuste *et al.*, 2004, 2005). In contrast, endocytosis signals may play a different role in infected macrophages where HIV assembles on intracellular membranes. Interestingly, the fact that SIV_{mac239}-carrying mutations in the GYxxØ motif is nonpathogenic, even though these proteins continue to undergo endocytosis through the activity of the second yet to be identified signal (Bowers *et al.*, 2000), suggests the GYxxØ signal has additional roles and that its genetic conservation throughout the primate lentiviruses reflects a key functional importance. Further work analyzing the activities of the viral trafficking motifs in cellular systems and animal models will indicate their role in viral assembly and in pathogenesis.

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