Interaction of Endocytic Signals from the HIV-1 Envelope Glycoprotein Complex with Members of the Adaptor Medium Chain Family

Hiroshi Ohno, Ruben C. Aguilar, Marie-Christine Fournier, Silke Hennecke,* Pierre Cosson,* and Juan S. Bonifacino

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and *Basel Institute for Immunology, Grenzacherstrasse 487, Postfach CH-4005, Basel, Switzerland

Received June 5, 1997; returned to author for revision July 18, 1997; accepted September 9, 1997

The envelope glycoprotein (Env) complex of HIV-1 undergoes rapid internalization from the plasma membrane of human cells by virtue of a tyrosine-based endocytic signal (RQGYSP, residues 704–710) in the cytosolic tail of the protein (J. F. Rowell et al., J. Immunol. 155, 473–488, 1995). Here we demonstrate that this tyrosine-based signal interacts with the $\mu_2$ (medium) chain of the AP-2 clathrin-associated adaptor, a protein complex involved in endocytosis of cell surface receptors. The same signal is also capable of interacting with two other members of the adaptor medium chain family, $\mu_1$ and $\mu_3A$, which are components of the AP-1 and AP-3 adaptor complexes, respectively. Interactions with $\mu_1$ and $\mu_3A$ might be responsible for the targeting of the internalized envelope glycoprotein to lysosomes or to the basolateral plasma membrane of polarized epithelial cells. A second potential tyrosine-based signal (LFSYHRL, residues 760–766) also interacts with $\mu_1$, $\mu_2$, and $\mu_3A$, although it is less important for internalization in vivo probably due to its position within the cytosolic tail. Overexpression of chimeric proteins having the HIV-1 Env cytosolic tail increases expression of the transferrin receptor on the cell surface, probably due to saturation of the cellular pool of $\mu_2$ by the overexpressed proteins. These observations suggest that HIV-1 Env utilizes the protein sorting machinery of the host cells for internalization and sorting at various steps of the endocytic and biosynthetic pathways.

INTRODUCTION

The envelope glycoprotein (Env) complex of HIV-1 is a membrane-bound heterodimer composed of a transmembrane subunit (gp41) noncovalently associated to a peripheral surface subunit (gp120) (reviewed by Freed and Martin, 1995). The Env complex is primarily responsible for the specific binding of the virus to the target cells and for the fusion of the viral and target cell membranes. These processes involve binding of gp120 to CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and chemokine receptors (Alkhathib et al., 1996; Doranz et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragoni et al., 1996) on the surface of the target cells and membrane fusion mediated by gp41 (Kowalski et al., 1987; Freed et al., 1990). In addition, the Env complex determines the polarized budding of HIV-1 from the basolateral plasma membrane of epithelial cells (Lodge et al., 1994, 1997) in a process that may involve interactions with the HIV-1 matrix protein (Freed and Martin, 1995; Yu et al., 1993; Cosson, 1996). The Env complex has also been implicated in causing some of the cytopathic effects of the virus (York Higgins et al., 1990; Laurent-Crawford et al., 1993; Cao et al., 1994).

The intracellular trafficking of the HIV-1 Env complex in the host cells has been the subject of considerable interest since the discovery of the virus. The complex is synthesized in the ER as a high mannose glycoprotein precursor, gp160, which oligomerizes, undergoes processing of its oligosaccharide chains, is cleaved into gp120 and gp41 by furin or a related endopeptidase, and is eventually transported to the plasma membrane (reviewed by Freed and Martin, 1995). While many aspects of the biogenesis of the Env complex are similar to those of other host cell proteins that are transported to the plasma membrane, the trafficking of the Env complex has several interesting features that deserve special consideration. First, much of the newly synthesized gp160 precursor protein is retained in the ER (Willey et al., 1988). Second, some of the surface gp120 is shed into the extracellular medium leaving only gp41 attached to the plasma membrane (Schneider et al., 1986). Third, the gp120–gp41 complex undergoes internalization from the plasma membrane (Rowell et al., 1995). All of these processes contribute to reducing the amount of gp120–gp41 that is available for incorporation into budding viral particles.

The inefficiency with which the Env complex is expressed at the cell surface does not present an obstacle for propagation of the infection but might in fact provide means of regulating virus assembly or virus-host interactions. Such a view has been derived from analyses of the rapid internalization of the Env complex from the...
cell surface. Recent studies have demonstrated that the cytosolic domain of gp41 contains conserved endocytic signals that mediate internalization of the Env complex when it is expressed in the absence of other HIV-1-encoded proteins (Rowell et al., 1995; Egan et al., 1996). Two potential endocytic signals are contained within the sequences RQGYSPL (residues 704–710) and LFSYHRL (residues 760–766), both of which conform to the YXXØ motif for tyrosine-based sorting signals present in many endocytic receptors (Trowbridge et al., 1993; Marks et al., 1997) (Fig. 1a). Similar signals have been implicated in the rapid endocytosis of the Env complex of simian immunodeficiency virus, a relative of HIV-1 (Lambert et al., 1995; Sauter et al., 1993; Marks et al., 1996). Studies by Rowell et al. (1995) have demonstrated that the membrane-proximal signal (RQGYSPL) in the HIV-1 gp41 cytosolic tail is particularly important for rapid endocytosis of the Env complex. Expression of other HIV-1 proteins during infection abolishes rapid internalization of the Env complex, a phenomenon that has been attributed to masking of the signals by the HIV-1 matrix protein (Egan et al., 1996). The regulated endocytosis of the Env complex has been proposed to represent a control mechanism that ensures that only those Env complexes that interact with the matrix protein remain at the cell surface and are incorporated into virions (Egan et al., 1996).

Despite the growing interest in the endocytosis of the cell surface Env complex, little is known about the cellular factors that control its internalization. The resemblance of the Env endocytic signals to other signals found in host cell proteins suggests that they may share the same endocytic machinery. In the present study we demonstrate that this is indeed the case. We show that overexpression of proteins having the tyrosine-based signals of the Env complex saturates the intracellular sorting machinery and, as a consequence, increases the surface expression of the transferrin receptor (TfR), a protein that is normally internalized by virtue of a tyrosine-based signal (Collawn et al., 1990; McGraw and Maxfield, 1990). Furthermore, we demonstrate that the tyrosine-based signals of the HIV-1 Env complex bind to the μ2 chain of clathrin-associated adaptor complex AP-2, a protein that was previously implicated in the endocytosis of other cellular proteins (Ohno et al., 1995, 1996; Boll et al., 1996). Finally, we show that the same signals interact with two other members of the adaptor medium chain family, μ1 and μ3A, albeit with lower avidity. The significance of this latter finding is unclear at the present time, although it may be related to the sorting of the Env complex at intracellular sites, such as it would be required to target it to lysosomes (Willey et al., 1988; Bird et al., 1990) or to the basolateral membrane of polarized epithelial cells (Lodge et al., 1994, 1997). The identification of μ2 as a recognition molecule for the endocytic signals of the Env complex should allow more detailed analyses of the molecular mechanisms that govern its expression at the cell surface.

MATERIALS AND METHODS

Recombinant DNA constructs

The Tac-Env chimeric constructs TE-11 and TE-16 (Fig. 1B) were made by ligation of PCR-amplified DNA fragments into pCDM8.1-Tac, as previously described (Humphrey et al., 1993). The constructs TE-17 and TE-18 were similarly made and cloned into pcDNAI/Amp (Invitrogen, San Diego, CA). The TTMb construct was described by Marks et al. (1996) and the GAL4ad-μ1, GAL4ad-μ2, and GAL4ad-μ3A constructs were described by Ohno et al. (1996). The constructs GAL4bd-RQGYSPL, -RQGASPL, -LFSYHRL, and -LFSAHRL were made by substitution of the ASYQRL sequence from a GAL4ad-TGN38 construct (Ohno et al., 1996). DNAs encoding these proteins were made by PCR and ligated into the EcoRI and Sall sites of the vector pGBT9 (Clontech, Palo Alto, CA). The sequence of all the recombinant constructs was verified by DNA sequencing.

Cell culture and transfection

HeLa cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Biofluids, Rockville, MD), and 50 μg/ml gentamicin (Bio Whitaker, Walkensville, MD) (regular medium). For transient transfections (for further details, see Marks et al., 1996), HeLa cells were seeded onto 10-cm culture dishes (Falcon 3003, Becton–Dickinson, Lincoln Park, NJ) in 10 ml of regular medium. Three to 5 h later, calcium phosphate precipitates containing 10 μg of DNA in 1 ml were added to the cells. The next day, the medium was replaced with fresh culture medium.

Immunofluorescence microscopy

Immunofluorescence microscopy of fixed-permeabilized HeLa cells and antibody internalization microscopy experiments were done as previously described (Humphrey et al., 1993; Marks et al., 1996). Antibodies used in these experiments were: 7G7, a mouse monoclonal antibody directed to a luminal epitope of the human Tac antigen (American Type Culture Collection, Rockville, MD); R2873, a rabbit polyclonal antibody to Tac produced in our laboratory; B3/25, a mouse monoclonal antibody to the human transferrin receptor (Boehringer Mannheim, Indianapolis, IN); H4A3, a mouse monoclonal antibody to

2 Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain (leucine, isoleucine, phenylalanine, methionine, or valine).
human lamp-1 (Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD), and rhodamine-conjugated goat anti-rabbit IgG, rhodamine-conjugated donkey anti-mouse IgG, and fluorescein-conjugated donkey anti-rabbit IgG (all from Jackson ImmunoResearch, West Grove, PA).

Overexpression-saturation assays

Overexpression–saturation assays were done as described previously (Marks et al., 1996). Transfected HeLa cells were released from tissue culture dishes by incubation with 20 mM EDTA in PBS, harvested, stained with phycoerythrin-conjugated anti-Tac (CD25) monoclonal antibody (mAb) and fluorescein isothiocyanate (FITC)-conjugated anti-transferrin receptor (TR) (CD71) mAb or FITC-conjugated anti-HLA mAb (Immunotech, Westbrook, ME), and analyzed using a Becton–Dickinson FACScan (Mountain View, CA) and the CellQuest 1.2 program.

Yeast culture, transformation, and two-hybrid assays

The yeast strain HF7c (Clontech, Palo Alto, CA) was maintained on YPD agar plates. Transformation was done as described in the instructions for the MATCHMAKER two-hybrid kit (Clontech). For colony growth assays, HF7c transformants were streaked onto plates lacking leucine, tryptophan, and histidine and incubated at 30°C for 4–5 days. Filter assays for β-galactosidase activity were done according to the instructions for the MATCHMAKER two-hybrid kit.

Quantitative growth assays were carried out as described by Aguilar et al., (1997). Briefly, three to five colonies of each HF7c transformant were added to 20 ml of liquid medium lacking leucine, tryptophan, and histidine (–His medium) and grown at 30°C to 1.0–1.2 OD_{600} units/ml. 3 × 10^{-3} OD_{600} units (~3 µl) were inoculated into 20 ml of –His medium in the absence or presence of several concentrations of 3AT (3-amino-1,2,4-triazole, Fluka Chemie AG, Buchs, Switzerland); after 2 days at 30°C the OD_{600} of triplicates was measured. The results were expressed as fractions of the signal obtained in the absence of 3AT (control).

β-galactosidase assays of HF7c transformants (five to seven colonies/culture) in liquid culture were done using a chemiluminescent β-galactosidase assay kit (Clontech). Briefly, yeast cells were resuspended at 10 OD_{600} units/ml in lysis buffer (100 mM sodium phosphate, 1 mM DTT, pH 7.4) and broken by vortexing in the presence of glass beads. After centrifugation at 4°C for 15 min in a microfuge, 10–50 µl of the lysate was added to 200 µl of kit reaction buffer, incubated for 1 h at room temperature, and the light emission recorded at 5-s integrals in a tube luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). Results were normalized by protein concentration and expressed as the mean ± SD of three independent determinations.

RESULTS

The cytosolic tail of the HIV-1 Env complex has sufficient information for internalization from the plasma membrane

Previous studies of the internalization of the HIV-1 Env complex were done using the full-length gp160 precursor protein as the substrate for mutational analyses (Rowell et al., 1995; Egan et al., 1996). We wished to examine whether the cytosolic tail of gp41 was sufficient for internalization from the plasma membrane. In addition, we wanted to compare the relative contributions to internalization of the two cytosolic tyrosine-based signals (RQGYSPL and LFSYHRL, see Fig. 1A) independent of other complicating factors derived from the use of the full-length Env protein (i.e., inefficient exit from the ER, gp120 shedding). For these reasons, we constructed chimeric proteins having the luminal and transmembrane domains of the Tac antigen, a plasma membrane protein often used as a reporter (Humphrey et al., 1993; Voorhees et al., 1995; Marks et al., 1996), and the cytosolic domain of the HIV-1 tail (Fig. 1B). In addition to a chimeric construct having both tyrosine-based signals intact (TE-11), we designed constructs in which either one (TE-16 and TE-17) or both (TE-18) of the critical tyrosines were mutated to alanines (Fig. 1B).

The constructs were expressed by transient transfection into HeLa cells and examined for their ability to mediate internalization of antibodies to the Tac luminal domain by immunofluorescence microscopy (Fig. 2), as previously described (Humphrey et al., 1993; Voorhees et al., 1995; Marks et al., 1996). We observed that whereas the normal Tac antigen remained at the cell surface (Fig. 2A), TE-11 (bearing the full-length Env tail) was internalized into vesicular structures (Figs. 2B and 2C). Some of these vesicles contained the transferrin receptor (Figs. 2C and 2D), indicating that they corresponded to early endosomes. In other vesicles, TE-11 colocalized with the lysosomal membrane protein lamp-1 (Figs. 2E and 2F), suggesting that a fraction of the internalized TE-11 chimera was transported into late endosomes or lysosomes. Mutation of the membrane-proximal tyrosine (Y^{707} within the sequence RQGYSPL) abrogated rapid internalization, as evidenced by the staining pattern similar to normal Tac (Fig. 2G, TE-16). On the other hand, mutation of the membrane-distal tyrosine (Y^{763} within the sequence LFSYHRL) slightly increased cell surface staining but did not abolish internalization of the anti-Tac antibody (Fig. 2H, TE-17). As expected, mutation of both tyrosines prevented internalization of the chimeric protein (Fig. 2E, TE-18).

The above results demonstrated that the cytosolic do-
### FIG. 1

(A) Potential tyrosine-based sorting signals present in the cytosolic domain of the HIV-1 Env complex and their correspondence to the YXXØ consensus motif. Numbers of the first and last amino acid residues of the sequences, relative to the full-length gp160, are indicated. (B) Schematic representation of the constructs used in this study. All the constructs had the luminal and transmembrane domains of the Tac antigen. Tac-Env chimeras have the cytosolic domain of wild-type or mutant HIV-1 Env sequences, as shown in the figure.

<table>
<thead>
<tr>
<th></th>
<th>Lumen</th>
<th>Membrane</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tac</td>
<td></td>
<td></td>
<td>COOH</td>
</tr>
<tr>
<td>TE-11</td>
<td>RQGYSPL</td>
<td>LFSYHRL</td>
<td>COOH</td>
</tr>
<tr>
<td>TE-16</td>
<td>RQGASPL</td>
<td>LFSYHRL</td>
<td>COOH</td>
</tr>
<tr>
<td>TE-17</td>
<td>RQGYSPL</td>
<td>LFSAHRL</td>
<td>COOH</td>
</tr>
<tr>
<td>TE-18</td>
<td>RQGASPL</td>
<td>LFSAHRL</td>
<td>COOH</td>
</tr>
</tbody>
</table>

**Tac**

**Env**

Overexpression of a chimera having the cytosolic tail of the HIV-1 Env complex increases surface expression of the TR.

The tyrosine-based endocytic signals in the HIV-1 Env tail (i.e., RQGYSPL and LFSYHRL) fit the YXXØ consensus motif for endocytic signals (Marks et al., 1997). Similar signals are present in the cytosolic tails of many cell surface receptors such as the TR (i.e., LSYTRF). In order to examine if the Env complex shares the same internalization machinery with cellular receptors, we used an overexpression-saturation assay described previously (Marks et al., 1996). In this assay, proteins having putative endocytic signals are overexpressed by transient transfection into HeLa cells and the surface expression of an endogenous protein having a tyrosine-based endocytic signal (in this case the TR) is measured by FACS analysis. Approximately 70% of the TR is intracellular at steady state (Harford et al., 1991), mainly due to the activity of its tyrosine-based endocytic signal that targets it to endosomes. If the overexpressed protein and the TR compete for binding to components of the internalization machinery, then one would expect to see decreased internalization of the TR and, consequently, increased accumulation of the TR at the cell surface.

**Figure 3** shows the results of a typical overexpression-saturation experiment. Overexpression of the normal Tac antigen, which does not have any endocytic signals, did not change the amount of TR at the cell surface, as evidenced by the vertical distribution of fluorescence intensities in the FACS profile (Fig. 3A). In contrast, overexpression of TTMb, a chimeric protein having a tyrosine-based endocytic signal (i.e., SSYTPL, Marks et al., 1996), yielded a diagonal FACS profile, indicating that expression of TR on the cell surface increased proportionally to the expression of TTMb (Fig. 3B). Finally, overexpression of the TE-11 chimera, which has the cytosolic tail of the HIV-1 Env complex, also led to increased surface TR, yielding a diagonal distribution of fluorescence intensities (Fig. 3C). Neither of the three overexpressed constructs had any effect on the surface expression of a class I MHC molecule (HLA) (notice the vertical FACS profile in Figs. 3D–3F), a protein that is not rapidly internalized from the cell surface. These observations suggest that the TE-11 chimera and the TR compete for the same internalization machinery.

Interaction of the HIV-1 Env endocytic signals with the $\mu_2$ chain of the AP-2 complex

Recently, tyrosine-based sorting signals, including that of the TR, have been shown to interact with the $\mu_2$ chain...
FIG. 2. Analysis of the internalization of Tac and Tac-Env chimeras by immunofluorescence microscopy. Live HeLa cells expressing Tac or Tac-Env chimeras were incubated with either a mouse monoclonal antibody (7G7 in A, B, G, H, I) or a rabbit polyclonal antibody (C - F) to the luminal domain of Tac for 2 h at 37°C. In A, B, G, H, and I, cells were fixed and the internalized antibody was detected by incubation with Cy3-conjugated anti-mouse IgG antibody. The presence of proteins at the plasma membrane was evidenced by staining of the outline of the cells, including many cell surface projections (A, G, I). Internalized proteins were detected in intracellular vesicles (B, H). In C - F, fixed-permeabilized cells were incubated with mouse monoclonal antibodies to either the TfR (C, D) or lamp-1 (E, F), followed by incubation with fluorescein-conjugated anti-rabbit and Cy3-conjugated anti-mouse IgG antibodies. The internalized TE-11 protein displayed partial colocalization with both the TfR (C, D) and lamp-1 (E, F). Arrows point to vesicular structures containing TE-11 and either TfR or lamp-1.
FIG. 3. Overexpression of a Tac-Env chimera increases surface expression of the Tfr. HeLa cells were transiently transfected with constructs encoding Tac (A, D), TTMb (B, E), or TE-11 (C, F), under conditions that result in overexpression of the proteins (Marks et al., 1997). Two days after transfection, the cells were analyzed by FACS for surface expression of two endogenous proteins, the Tfr (A–C) and a class I MHC antigen (HLA, D–F). The graphs represent the fluorescence intensity of the transfected gene products (y axis) as a function of the fluorescence intensity of the endogenous proteins (x axis). A vertical distribution of fluorescence intensities (as in A) means that overexpression of the transfected gene product has no effect on the surface expression of the endogenous protein. In contrast, a diagonal distribution (as in B and C) is indicative of displacement of the endogenous protein to the cell surface upon overexpression of the transgene. Notice that overexpression of TE-11 increases surface Tfr levels (C).

of the clathrin-associated adaptor complex AP-2 (Ohno et al., 1995, 1996; Boll et al., 1996). This complex is localized to clathrin-coated areas of the plasma membrane, where endocytosis of cell surface proteins takes place. The demonstration that a construct having the Env tail competes with the Tfr for the internalization machinery (Fig. 3) suggested that the endocytic signals of the Env complex might similarly interact with μ2. To address this issue directly, we used a yeast two-hybrid system (Ohno et al., 1995, 1996). To this end, the tyrosine-based signals of the Env complex (RQGYSPL and LFSYHRL) and the corresponding tyrosine-mutated versions of the signals (RQGASPL and LFSAHRL) were fused to the carboxy-terminus of the GAL4 DNA binding domain. These constructs were coexpressed in yeast cells with μ2 fused to the carboxy-terminus of the GAL4 transcription activation domain. The occurrence of interactions between the signals and μ2 was determined by activation of the HIS3 gene, which confers the ability to grow in minimal medium lacking histidine (−His), and the LacZ gene, which leads to expression of β-galactosidase activity. Both assays demonstrated that the RQGYSPL and LFSYHRL signals interacted with μ2 in a tyrosine-dependent fashion (Fig. 4). This observation indicated that both signals are potentially capable of binding to recognition molecules involved in internalization. The fact that the RQGYSPL signal is more potent in vivo (Rowell et al., 1995; this study) may be due to its more favorable context within the cytosolic tail, a factor that has recently been found to be critical for the function of other tyrosine-based signals (Ohno et al., 1996; Rohrer et al., 1996).

Interaction of the HIV-1 Env endocytic signals with other members of the adaptor medium chain family

The protein μ2 is a member of a growing family of adaptor medium chains that also includes μ1 (Nakayama et al., 1991) and μ3A (originally known as p47A, Pevsner et al., 1994). μ1 is a component of the TGN-localized AP-1 complex (Robinson, 1987; Ahle et al., 1988) and μ3A is a component of the TGN/endosome-localized AP-3 complex (Simpson et al., 1996, 1997; Dell’Angelica et al., 1997a, 1997b; Ooi et al., 1997). Both AP-1 and AP-3 are thought to mediate intracellular sorting events dependent on tyrosine-based sorting signals. The fact that all the μ chains are structurally related prompted us to test whether μ1 and μ3A would also be capable of interacting with the tyrosine-based signals of the Env complex. Yeast two-hybrid assays were performed by testing for growth on histidine-deficient plates. These assays revealed that both the RQGYSPL- and LFSYHRL-containing constructs supported growth on histidine-deficient (−His) plates when coexpressed with either of the three medium chains (Fig. 5); all interactions were dependent on the presence of the critical tyrosine residues (Fig. 5).

To compare the relative avidity of the interaction of the signals with each medium chain, we performed two types of quantitative analyses. The first analysis consisted of
determining the growth of cotransformed yeasts in histidine-deficient liquid medium in the presence of varying concentrations of 3-amino-1,2,4-triazole (3AT), a competitive inhibitor of the HIS3 gene product. In this assay, the higher the avidity of the signal for the medium chain, the higher the concentration of 3AT needed to inhibit growth (Fig. 6A). The dose of 3AT that inhibits 50% of the growth (IC₅₀) is a good indicator of the relative avidity of an interaction (Fig. 6B). The second assay consisted of measuring β-galactosidase activity of the cotransformed yeast cells (Fig. 7). Both assays demonstrated that μ2 exhibited the highest apparent avidity for both Env complex signals (Figs. 6 and 7). Interactions with μ1 and μ3A were significantly weaker, although they were all above background levels. Taken together, these observations indicate that the tyrosine-based signals of the HIV-1 Env complex are capable of interacting with μ1, μ2, and μ3A, although interactions with μ2 are the strongest.

**DISCUSSION**

In this study, we have used a chimeric protein approach to demonstrate that the cytosolic tail of the HIV-1 Env gp41 subunit has sufficient information for internalization from the cell surface and that a tyrosine-based signal (RQGYSPL) is the main determinant of endocytosis. These findings confirm and extend previous observations by Siliciano and colleagues (Rowell et al., 1995; Egan et al., 1996) who first established the importance of the RQGYSPL sequence in endocytosis of the complete Env complex. While the cytosolic tail of gp41 alone can confer the ability to undergo internalization, we expect the RQGYSPL sequence to be even more effective for internalization in the context of the whole Env complex. This is due to the fact that the Env complex is an oligomer (Schawaller et al., 1989; Earl et al., 1991; Willey et al., 1991), in contrast to the reporter protein used in
it is currently not feasible to establish a priori whether a particular YXXØ-type signal will interact with \( \mu_2 \), thus the need to demonstrate it for the HIV-1 Env signals. The relatively strong avidity of the two HIV-1 Env signals for \( \mu_2 \) might be explained by the presence of a proline at position +2 from \( Y^{707} \) in the RQGYSPL signal and an arginine at position +2 from \( Y^{763} \) in the LFSYHRL signal. Either proline or arginine at position +2 from a tyrosine have been shown to favor interaction with \( \mu_2 \) (Ohno et al., 1996; Boll et al., 1996).

While both HIV-1 Env tyrosine-based signals are intrinsically capable of interacting with \( \mu_2 \), previous analyses of the effects of mutations on internalization in intact cells have established that the membrane-proximal \( Y^{707} \), but not the membrane-distal \( Y^{763} \), is required for rapid endocytosis of the Env complex (Rowell et al., 1995). We have confirmed these observations by performing the same mutations in the context of a Tac-Env chimera (TE-11). Similarly, studies of SIV have shown that mutation of a membrane-proximal tyrosine \( Y^{723} \) situated within the sequence RQGYRPV abrogates rapid internalization of the SIV Env complex from the cell surface (LaBranche et al., 1995; Sauter et al., 1996). These observations suggest that the position of tyrosine-based signals within the cytosolic tail is an important determinant of function. The critical role of \( Y^{707} \) in internalization might be due to its proximity to the membrane, where it may interact more effectively with the adaptor complexes. Rohrer et al. (1996) have demonstrated that an appropriate spacing

![Graph A](image1.png)

**FIG. 6.** Analysis of the interaction of tyrosine-based signals of HIV-1 Env with members of the adaptor medium chain family using a quantitative growth assay. (A) Yeast cells cotransformed with different combinations of constructs encoding the RQGTSPL or LSFYHRL signals and the \( \mu_1 \), \( \mu_2 \), or \( \mu_3A \) chains were analyzed for growth in liquid minimal medium lacking histidine in the presence of different concentrations of the histidine-biosynthesis inhibitor, 3AT. The concentration of yeast cells was determined spectrophotometrically by measuring the optical density at 600 nm. (B) The concentration of 3AT that causes 50% inhibition of growth (IC\(_{50}\)) was calculated from the data shown in A by performing nonlinear regression analysis.

![Graph B](image2.png)

**FIG. 7.** Quantitative \( \beta \)-galactosidase analysis of the interaction of tyrosine-based signals from HIV-1 Env (RQGTSPL or LSFYHRL) with members of the adaptor medium chain family (\( \mu_1 \), \( \mu_2 \), or \( \mu_3A \)). \( \beta \)-galactosidase activity of cotransformed yeast cells grown in liquid culture was measured using a quantitative chemiluminescence assay. Results are expressed as the mean ± standard deviation of triplicate determinations (in relative light units, RLU) and represented on a logarithmic scale.
from the membrane is important for the activity of a tyrosine-based signal in lysosomal targeting. In addition, the sequence or conformational context of the signal might also affect its recognition. For instance, Y707 might be more accessible for interaction with the adaptors because of a favorable conformation of the surrounding sequences. The opposite might be the case for Y733, the recognition of which could be hindered by its conformational context.

Two other members of the adaptor medium chain family, µ1 and µ3A, have also been shown to interact with both tyrosine-based signals of the gp41 cytosolic tail. This is consistent with our previous findings that µ1 and µ3A share with µ2 the ability to bind tyrosine-based signals (Ohno et al., 1995, 1996; Dell’Angelica et al., 1997). However, the interactions of the HIV-1 signals with µ1 and µ3A are comparatively weak and it is currently unclear whether these interactions reflect a role for these proteins in the sorting of the Env complex in vivo. Nevertheless, an attractive possibility is that interactions with these chains might be responsible for the sorting of the HIV-1 Env complex at some intracellular site. The transport of a fraction of the internalized chimeric proteins to lysosomes (Fig. 2), for example, could be mediated by interaction with µ1 and/or µ3A. This lysosomal targeting could be related to the turnover of the Env complex (Wiley et al., 1988; Bird et al., 1990) or to the disposal of gp41 after shedding of gp120 (Schneider et al., 1986). Another possibility is that interactions with µ1 and/or µ3A could play a role in the targeting of the Env complex to the basolateral plasma membrane of polarized epithelial cells. In this type of cell, HIV-1 has been shown to be released from the basolateral surface, in a process that requires the cytosolic tail of the protein (Lodge et al., 1994). Strikingly, this process is dependent upon the integrity of Y707 (Lodge et al., 1997), the same residue involved in rapid internalization. Since sorting to the basolateral surface occurs at the TGN and/or endosomes (Matter and Mellman, 1994), we speculate that recognition of the tyrosine-based signal containing Y707 by µ1 or µ3A might be the event that determines basolateral targeting of the Env complex.

Tyrosine-based sorting signals similar to those described here are present in almost all isolates of HIV-1 and SIV (Meyers et al., 1993; Sauter et al., 1996). Similar signals are also present in the membrane proteins of other enveloped viruses such as bovine leukemia virus (Willems et al., 1995), vesicular stomatitis virus (Thomas and Roth, 1994), varicella-zoster virus (Zhu et al., 1996; Alconada et al., 1996), and alphavirus (Zhao et al., 1994). These signals have been implicated in various functions that are important in the life cycle of the viruses, including internalization, basolateral targeting, budding, and infectivity. Our finding that the tyrosine-based signals interact with members of the adaptor medium chain family suggest that these cellular proteins may play a general role in regulating the trafficking of viral envelope glycoproteins in infected cells.

ACKNOWLEDGMENTS

This project was funded by the NIH Intramural AIDS Targeted Antiviral Program. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-LaRoche & Co. We thank Ronald Willey, Chean Eng Ooi, Esteban Dell’Angelica, and Nathan Wolins for critical review of the manuscript.

REFERENCES


Deng, H. K., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhardt, M.,
Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. J. Virol. 70.


Thomas, D. C., and Roth, M. G. (1994). The basolateral targeting signal
in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. J. Biol. Chem. 269, 15732–15739.