

A Membrane-proximal Tyrosine-based Signal Mediates Internalization of the HIV-1 Envelope Glycoprotein via Interaction with the AP-2 Clathrin Adaptor*

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The envelope glycoprotein (Env) of human immunodeficiency virus, type 1 (HIV-1) undergoes rapid internalization after its transport to the cell surface. Env internalization is dependent upon information contained within the cytosolic domain of the protein. Here, we report that the cytosolic domain of Env binds specifically to the medium chain, $\mu 2$, of the clathrin-associated protein complex AP-2, as well as to the complete AP-2 complex. The Env cytosolic domain contains two highly conserved tyrosine-based motifs (Y^{712} SPL and Y^{768} HRL), both of which are capable of binding to $\mu 2$ when presented as short peptides. However, only the membrane-proximal motif Y^{712} SPL binds to $\mu 2$ and is required for internalization in the context of the whole cytosolic domain of Env. A glycine residue (Gly^{711}) adjacent to the Y^{712} SPL motif is also important for binding to $\mu 2$ /AP-2 and internalization. These observations suggest that the accessibility of the membrane-proximal Y^{712} SPL to $\mu 2$ /AP-2 determines its function as a signal for recruitment of HIV-1 Env into clathrin-coated pits and its ensuing internalization.

The envelope glycoprotein (Env) of human immunodeficiency virus, type 1 (HIV-1)¹ plays a critical role during the viral life cycle by mediating the attachment of virions to target cells and the fusion of viral and cellular membranes (1). Incorporation of Env, therefore, is essential for the formation of infectious viral particles. The cytosolic domain of the Env transmembrane subunit gp41 is the portion of the Env protein complex that is most likely to interact with the internal structural proteins of the virus (2–5). Although the cytosolic domain of Env is absolutely required for viral dissemination *in vivo* it seems to be dispensable for envelope incorporation into virions and, consequently, for viral replication *in vitro* (6). How newly assembled virions specifically acquire Env remains therefore largely unknown.

An intriguing characteristic of the Env proteins of HIV-1 and simian immunodeficiency virus is that they undergo rapid endocytosis after their transport to the cell surface (7, 8). As a

consequence, the internal structural proteins of these viruses need to compete with the internalization machinery of the cell in order to acquire Env (9).² Although the functional significance of this phenomenon is not understood, it is clear that Env behaves like other plasma membrane proteins that are rapidly internalized from the cell surface. Rapid internalization involves recruitment of plasma membrane proteins to clathrin-coated pits, a process that is mediated by interaction of endocytic signals found in the cytosolic domains of the proteins with the clathrin-associated adaptor complex AP-2 (10–12). The AP-2 complex consists of two large chains (α and $\beta 2$), a medium chain ($\mu 2$), and a small chain ($\sigma 2$). A direct interaction between $\mu 2$ and tyrosine-based sorting signals from the cytosolic domains of several cellular integral membrane proteins has been recently demonstrated (13–15).

To assess the diverse functions of the cytosolic domain of Env including its role in internalization from the plasma membrane, we are analyzing its interaction with cellular and viral proteins. Anti-Env antibodies allowed us to co-immunoprecipitate Env with the AP-2 complex from HIV-1-infected lymphocytes, demonstrating that these proteins associate *in vivo*. Using GST-Env tail fusion proteins, we then identified the $\mu 2$ chain of AP-2 as a protein that interacts with the cytosolic domain of Env. Binding of $\mu 2$ to the cytosolic domain of Env was dependent on the presence and the context of a tyrosine-based sorting motif that is crucial for Env internalization (7), but it was also influenced by a glycine residue that had not previously been identified to be important for efficient endosomal sorting. Moreover, sequence as well as context dependence of $\mu 2$ binding to the cytosolic domain of Env was mimicked by the intact AP-2 complex.

The results presented here suggest that the glutathione S-transferase (GST) gene fusion system may be useful to analyze the interaction of the cytosolic domain of Env with other cellular or viral proteins. This system may also permit further dissection of $\mu 2$ functional regions and definition of the requirements for the binding of tyrosine-based sorting signals to adaptor complexes other than AP-2.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—All constructs used in the *in vitro* binding assays were made by ligation of polymerase chain reaction (Pwo DNA polymerase; Boehringer Mannheim)-amplified DNA fragments into pGEX-3X (Amersham Pharmacia Biotech). Primer sequences were as follows: 5'-AATCCCGGGATAGTTTTGCTGTAC-3' and 5'-CTAAG-ACCACCTTGCCACCCATCTTA-3'. The polymerase chain reaction products were phosphorylated and inserted into *Sma*I-linearized pGEX-3X. The expression vectors 3M9 for $\mu 2$, pcwt for HIV-1 Env, and the deletion mutants of Env have been described (13, 16, 17); the latter were kindly provided by D. Gabuzda and J. Sodroski.

Site-directed mutagenesis was performed either in the GST fusion

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¹ The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MESNa, 2-mercaptoethanesulfonic acid.

² S. Wyss and M. Thali, unpublished observation.

constructs or in the Env expression vectors using the Quick Change™ system (Stratagene). The following primers were used: R709A, 5'-GTGAATAGAGTTGCGCAGGGATATTCAC-3'; Q710A, 5'-GTGAATAGAGTTAGGGCCGGCTATTCACCATTATCG-3'; G711A, 5'-GAGTTAGG-CAGGCATATTCATTATCG-3'; Y712A, 5'-GAGTTAGGCAGGGA-GCTTCTCCATTATCG-3'; S713A, 5'-GAGTTAGGCAGGATATGCAC-CATTATCG-3'; P714A, 5'-GTTAGGCAGGATATTCAGCATTATCGT-TTC-3'; L715A, 5'-GGCAGGATATTCGCCGGCGTCGTTTCAGACC-3'; Y768A, 5'-GTGCCTCTCAGCGCTACCGCTTGAGAGAC-3'; Y768C, 5'-GTGCCTCTCAGCTGCCACCGCTTGAG-3'. Mutations were confirmed by DNA sequencing.

Co-immunoprecipitation of Env and AP-2—Chronically infected Jurkat cells were established by transfecting the cells with proviral DNA (HXB2 strain; Ref. 18). Cells surviving the acute phase of virus production were propagated for 4–6 weeks in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Virus production was monitored by p24 capture (Abbott) and by SDS-PAGE analysis of purified virions. To perform the immunoblot analysis, uninfected or infected cells were washed in PBS and then lysed in 500 μ l of a Triton X-100-based lysis buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 10 mM NaF, and 1 mM EDTA) essentially as described (19). Precleared lysates were preincubated for 1.5 h with 50 μ l of Protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). Env was precipitated using the anti-Env monoclonal antibody b12 (20), kindly provided by Dennis Burton, immobilized on Protein A-Sepharose CL-4B overnight at 4 °C. After SDS-PAGE separation, the precipitated material was transferred onto nitrocellulose membranes. Those membranes were then probed with Sigma antibodies 100/2 (anti- α -adapting) or 100/1 (anti- β -adapting). Bound antibodies were visualized after binding of secondary antibodies by enhanced chemiluminescence (Pierce).

Expression of Fusion Proteins—GST fusion proteins were produced in *Escherichia coli* strain TB1 and purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Precultures (4 ml of LB medium supplemented with 0.5 M D-sorbitol and 2.5 mM betaine) were grown overnight at 37 °C. They were transferred to 400 ml of LB medium supplemented with 1 M D-sorbitol and 2.5 mM betaine and grown for 8 h at 37 °C. Cultures were shifted to 25 °C and induced overnight with 0.1 mM Isopropyl- β -D-thiogalactoside. The purification on glutathione-Sepharose 4B beads was performed according to the manufacturer's instructions including a heat shock step with 50 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, and 2 mM ATP for 10 min at 37 °C.

Binding Assays—³⁵S]Methionine-labeled $\mu 2$ was prepared using a coupled transcription-translation system (TNT[®], Promega). Precleared *in vitro* translated $\mu 2$ (2 μ l) was incubated with 5 μ g of GST fusion protein in 500 μ l of binding buffer (0.05% (w/v) Triton X-100, 50 mM HEPES (pH 7.3), 0.1 mM CaCl₂, 2 mM MgCl₂, 100 mM KCl, 50 μ M dithiothreitol, 10% (v/v) glycerol, 0.1% bovine serum albumin) for 2 h at room temperature. Loading buffer was added to washed beads, and the samples were run on SDS-PAGE. The amount of $\mu 2$ bound to the different GST fusion protein mutants was determined by measuring the radioactivity of the signals using the Instant Imager™ system (Packard).

T cell lysate was prepared from Jurkat cells essentially as described (21). Briefly, the cells were washed three times with cytosol buffer (25 mM Hepes, pH 7.0, 125 mM CH₃COOK, 2.5 mM (CH₃COO)₂Mg, 1.0 mM dithiothreitol, 1 mg/ml glucose), and the resulting pellet was resuspended in an equal volume of cytosol buffer containing 1 mM phenylmethylsulfonyl fluoride. The cell suspension was frozen in liquid nitrogen, thawed on ice, and drawn five times through a 21-gauge syringe. After centrifugation for 30 min at 20,000 \times g, 4 °C, the supernatant was transferred to new tubes in 100- μ l aliquots and stored at -80 °C. Aliquots were precleared in 900 μ l of PBS containing 1.5 mg of GST proteins on Sepharose 4B beads at 35 °C for 30 min. 300 μ l of precleared lysate was incubated with 100 μ g of fusion protein on Sepharose 4B beads at 35 °C for 2 h. Beads were washed three times with PBS and boiled in gel sample buffer. Afterward, SDS-PAGE proteins were transferred to nitrocellulose membranes and probed with Sigma antibody 100/2 (anti- α -adapting) or 100/1 (anti- β -adapting). Bound antibodies were visualized after binding of secondary antibodies by enhanced chemiluminescence (Pierce).

Internalization Assay—Twenty-four hours after transfection with the Env expression plasmids, HeLa cells were metabolically labeled for 16 h with ³⁵S]cysteine (50 μ Ci/ml). The labeled cells were chilled on ice and biotinylated for 30 min in 1 ml of PBS containing 1.5 mg/ml NHS-SS-biotin (Pierce). After three washes with PBS, 50 mM glycine, the cells were returned to 37 °C for different periods of time to allow for endocytosis to occur. To remove biotin from surface proteins that had not been internalized, cells were again chilled on ice and incubated

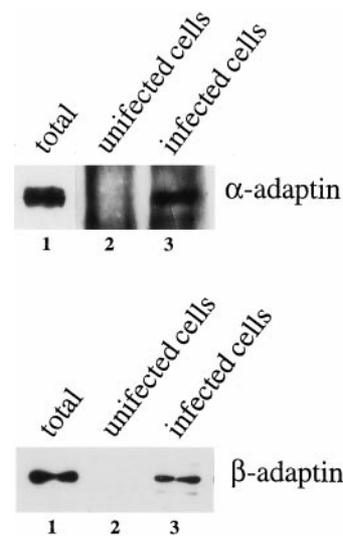


FIG. 1. HIV-1 Env and AP-2 associate in infected lymphocytes. Material immunoprecipitated with a monoclonal anti-HIV-1 Env antibody from uninfected (lanes 2) or HIV-1-infected (lanes 3) Jurkat cells was separated on SDS-PAGE. After transfer to nitrocellulose, the filters were probed with antibodies against the large chains of AP-2 (α -adapting and β -adapting, respectively). For comparison, an aliquot (1/50) of the T cell extract was loaded in lane 1.

twice for 15 min with 50 mM glutathione and for an additional 15 min with 60 mM 2-mercaptoethanesulfonic acid (MESNa). Free sulfhydryl groups were blocked by washing the cells three times with 50 mM glycine in PBS. The cells were then lysed in 1 \times radioimmune precipitation buffer, and viral proteins were immunoprecipitated with serum from an individual infected with HIV-1. Following immunoprecipitation, 90% of the material was boiled in 10% SDS, while the remaining 10% of the material was analyzed directly by SDS-PAGE to assess the amount of total Env in each sample. Biotinylated Env was recovered with streptavidin-agarose, followed by SDS-PAGE. The intensity of the Env signal was quantified using the Instant Imager™ system. The proportion of internalized Env was determined by subtracting for each time point the activity of the glutathione/MESNa-resistant biotin-tagged Env at 0 °C from the activity of the glutathione/MESNa-resistant biotin-tagged Env at 37 °C and dividing the remaining activity by one-hundredth of the activity of Env from cells that had not undergone reduction.

RESULTS

HIV-1 Env and AP-2 Associate in Infected Cells—The overall goal of our work is to identify proteins interacting with the cytosolic domain of HIV-1 Env. Since Env is rapidly retrieved from the cell surface after its transport to the plasma membrane (7), we tested whether Env interacts with the clathrin-associated protein complex AP-2. As shown in Fig. 1, AP-2 was detected in anti-Env immunoprecipitates from chronically infected lymphocytes but not in anti-Env immunoprecipitates from noninfected cells. A detailed analysis of the requirements for the observed Env-AP-2 interaction cannot be performed in infected cells, because many mutations in Env will affect the efficiency of viral replication and consequently also the levels of Env expression. Env-AP-2 interactions were therefore analyzed in more detail *in vitro*.

The Cytosolic Domain of HIV-1 Env Associates with $\mu 2$ —The cytosolic domain of Env contains two motifs that strongly resemble tyrosine-based endocytosis signals, one at position 712 (Y⁷¹²SPL) and the other at position 768 (Y⁷⁶⁸HRL) (Fig. 3A). Both motifs are well conserved among different strains of HIV-1 (18). Recently, the medium chain ($\mu 2$) of the clathrin-associated protein complex AP-2 was found to interact with tyrosine-based signals conforming to the canonical motif YXX \emptyset , where X is any amino acid and \emptyset is an amino acid with a bulky hydrophobic side chain (13–15). Therefore, we decided

to test whether the cytosolic domain of Env of HIV-1 associates with $\mu 2$. The cytosolic tail was expressed as a GST fusion protein (GST-EnvCD) in *E. coli*. The medium chain ($\mu 2$) of the clathrin-associated protein complex AP-2 was translated *in vitro* in a rabbit reticulocyte lysate. Fig. 2 shows that $\mu 2$ bound to GST-EnvCD but not to GST. For comparison, one-tenth of the *in vitro* translated $\mu 2$ is shown in the first lane. Typically, about 5–15% of the *in vitro* translated $\mu 2$ bound to GST-EnvCD.

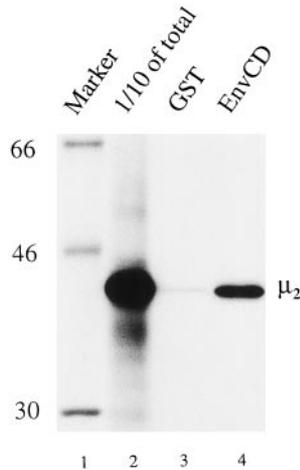


FIG. 2. Binding of the cytosolic domain of HIV-1 Env and $\mu 2$. *In vitro* translated [³⁵S]Met-labeled $\mu 2$ was incubated for 2 h with bacterially expressed GST (lane 3) or a fusion protein consisting of the whole cytosolic domain of the HIV-1 envelope glycoprotein fused to GST (GST-EnvCD, lane 4). Binding of $\mu 2$ to the immobilized recombinant proteins was analyzed on SDS-PAGE. For comparison, one-tenth of the *in vitro* produced $\mu 2$ was applied to lane 2.

Binding of $\mu 2$ to the Cytosolic Domain of Env Depends on the Presence and the Context of a Tyrosine-based Motif—To identify the binding site for $\mu 2$ in the cytosolic domain of Env, we tested the interaction of $\mu 2$ with various Env tail deletion mutants (Fig. 3B and data not shown). Deletions that did not affect the membrane-proximal tyrosine motif Y⁷¹²SPL had little or no effect on the association of $\mu 2$ with the cytosolic tail. Deletion of a region containing this membrane-proximal tyrosine-based motif, however, severely reduced the binding of $\mu 2$ to the cytosolic tail. In contrast, a deletion of region containing the motif Y⁷⁶⁸HRL had less of an effect. A more detailed analysis of the requirements for $\mu 2$ binding was then performed using mutants where single amino acids were mutated to alanine (Fig. 3C). As expected from the results shown in Fig. 3B, mutation of Tyr⁷⁶⁸ had very little impact on the binding of $\mu 2$ to the cytosolic domain of Env. Similarly, substitutions of amino acids Arg⁷⁰⁹, Gln⁷¹⁰, or Ser⁷¹³, respectively, by alanine influenced binding of $\mu 2$ only marginally. In contrast, binding of $\mu 2$ was decreased by mutation of Gly⁷¹¹ or Pro⁷¹⁴ and even more dramatically by mutation of Tyr⁷¹² or Leu⁷¹⁵. Thus, residues in the membrane-proximal tyrosine-based motif but not in the membrane-distal one are critical for the binding.

Although the specificity of the binding of $\mu 2$ to the cytosolic domain of Env of HIV-1 was apparent from the effects on binding of the deletion mutants and single amino acid substitutions, we corroborated these results by testing the ability of different peptides to inhibit the binding of $\mu 2$ to EnvCD. If protein-protein complexes were allowed to form in the presence of the peptide RRQGYSP, binding of $\mu 2$ was inhibited in a dose-dependent manner. No inhibition was observed if binding was performed in the presence of the peptide RRQGASPL, further confirming the specificity of this interaction (Fig. 4).

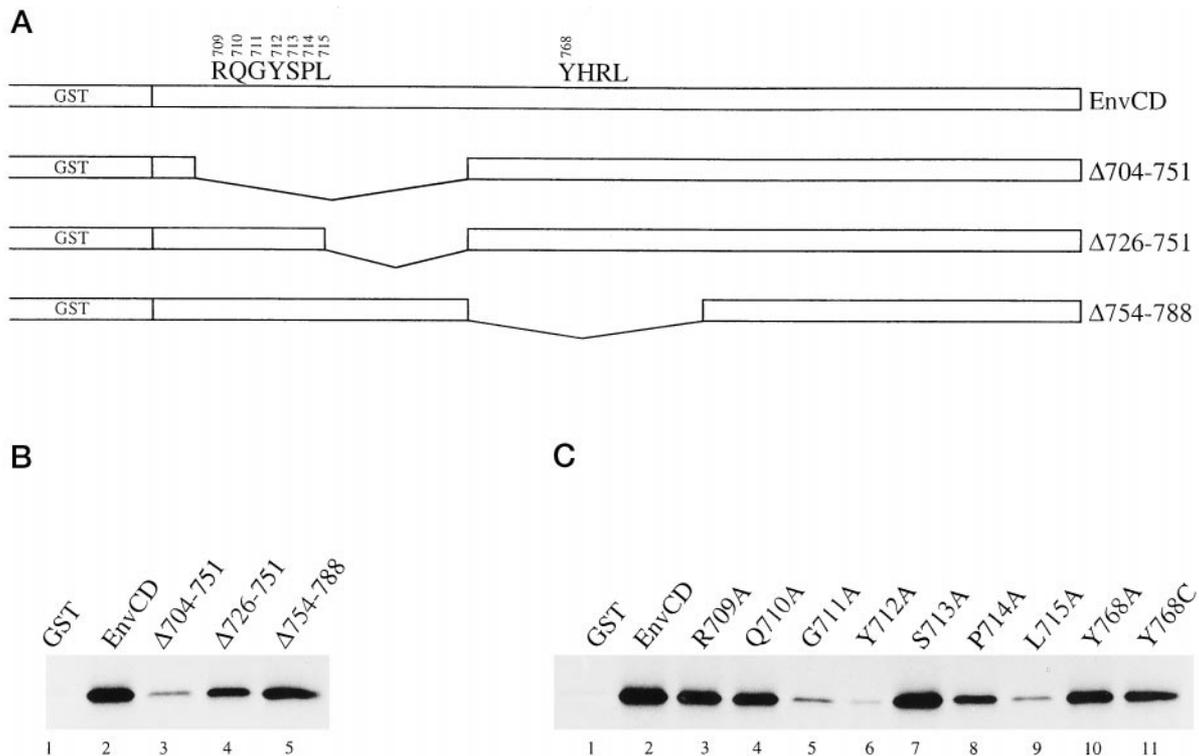


FIG. 3. The membrane-proximal tyrosine-based signal is critical for the binding of $\mu 2$ to the cytosolic domain of HIV-1 Env. *A*, schematic drawing of different fusion constructs that were analyzed for $\mu 2$ binding. The amino acid sequence of the two tyrosine-based motifs in the cytosolic domain of Env as well as their position within the Env tail is shown. *B*, binding of wild-type cytosolic tail of Env or the different deletion mutants to $\mu 2$. Binding assays were performed as described in Fig. 2. *C*, binding of the different mutants to $\mu 2$. A representative polyacrylamide gel is shown. For quantitation, the amount of bound $\mu 2$ was analyzed as described under "Experimental Procedures." The amount of $\mu 2$ bound to the mutants, expressed as the percentage of $\mu 2$ bound to the wild-type cytosolic domain of Env (\pm S.D.), was as follows: EnvCD, 100; R709A, 83.6 (\pm 24.7); Q710A, 90.3 (\pm 31.0); G711A, 14.2 (\pm 2.7); Y712A, 6.0 (\pm 6.5); S713A, 125.7 (\pm 35); P714A, 41.3 (\pm 9.2); L715A, 5.6 (\pm 6); Y768A, 93.0 (\pm 51); Y768C, 77.8 (\pm 7.1). Data are from five experiments.

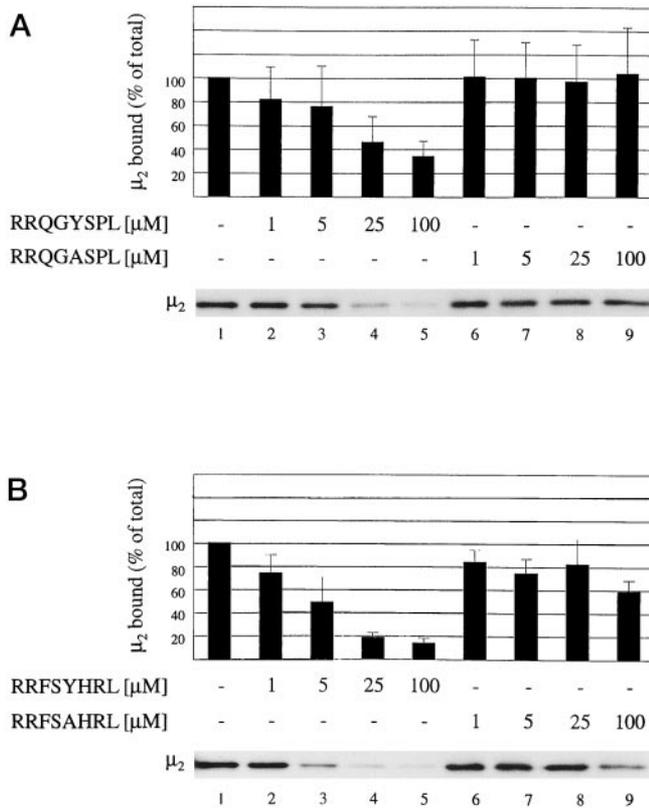


FIG. 4. Binding of $\mu 2$ is inhibited by peptides conforming to either of the two tyrosine-based signals. Increasing amounts of the wild-type or the tyrosine-mutated version of peptides conforming to either the membrane-proximal (A) or the membrane-distal (B) tyrosine-based signal were added to the binding reaction. Binding of $\mu 2$ to the cytosolic domain of Env was assessed and quantified as described in Figs. 2 and 3. A representative polyacrylamide gel is shown. The indicated amounts of the peptides were added just prior to the start of the binding reaction. Data are from five experiments.

Most interestingly, the peptide RRFSYHRL, the sequence of which conforms to the distal tyrosine-based signal, also interfered with the binding of $\mu 2$, while the tyrosine-mutated version of the same peptide did not. Thus, while the sequence YHRL within the context of the complete cytosolic domain of Env contributed very little to the binding of $\mu 2$ (see Fig. 3), it still was a potent $\mu 2$ binding element in the form of a small peptide.

Association of the Intact AP-2 Complex with the Cytosolic Domain of Env Shows the Same Sequence Requirements as Env- $\mu 2$ Binding—To test whether the same region of the cytosolic domain of Env important for $\mu 2$ binding represents the binding motif for intact AP-2 complexes, beads coated with some of our GST-EnvCD fusion proteins were incubated with cytosol of T cells and washed three times, and bound material was eluted. After SDS-PAGE, immunoblotting was performed using antibodies against either of the two large chains of the AP-2 complex (Fig. 5). Whereas the wild-type EnvCD, as well as the Y768C mutant, precipitated α and β -adapain, the Y712A mutant did not. Substitution of Gly⁷¹¹ for alanine, however, still allowed for the precipitation of some α - and β -adapain. These results demonstrate that the association of not only the isolated medium chain $\mu 2$ but also the intact AP-2 complex with the cytosolic domain of Env is critically dependent on the integrity of the membrane-proximal sequence Y⁷¹²SPL. In addition, Gly⁷¹¹ also contributes to the binding of EnvCD to AP-2.

Binding of $\mu 2$ /AP-2 to the Cytosolic Domain of Env Correlates with Env Internalization—The functional importance of some residues in the cytosolic domain of Env in its sorting to

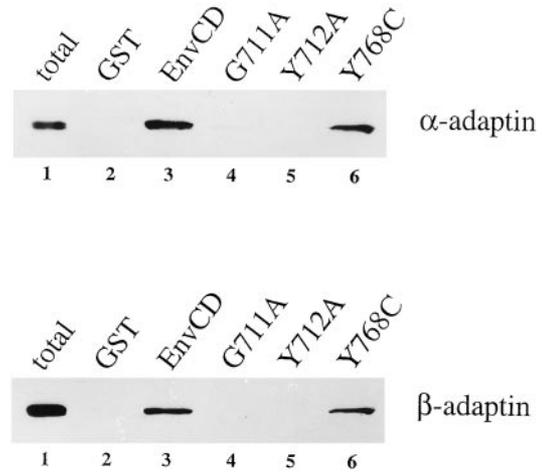


FIG. 5. Recruitment of intact AP-2 complexes by the cytosolic domain of Env. Immobilized GST-cytosolic domains of Env fusion proteins were incubated with T cell cytosol, and bound material was separated on SDS-PAGE. The effect of mutations in the Env tail on AP-2 recruitment was assessed by immunoblotting using antibodies against the large chains of AP-2 (α -adapain, β -adapain) as in Fig. 1. For comparison, an aliquot of the T cell extract was loaded in lane 1. The mutants used are described in Fig. 3.

intracellular compartments was assessed using a biochemical internalization assay (see “Experimental Procedures”). None of the deletion mutants was tested for endocytosis, because the cytosolic domain of Env partially overlaps with the viral Rev function, which is necessary for Env expression in intact cells (22). Fig. 6 shows the results of our internalization studies for three of the mutants where single amino acids had been substituted. The same mutations in the cytosolic domain of Env that led to a decrease in association with $\mu 2$ and the intact AP-2 complex (see Figs. 3C and 5, respectively) also reduced Env internalization. On the other hand, mutation of Tyr⁷⁶⁸, which had little effect on $\mu 2$ /AP-2 binding, did not affect endocytosis of Env. Using a fluorescence-activated cell sorting-based endocytosis assay, Siliciano and colleagues (7) had already demonstrated that the Tyr⁷¹² in the membrane-proximal motif but not Tyr⁷⁶⁸ in the membrane-distal motif was important for internalization of Env from the cell surface. Our data thus confirm those results and also show that glycine at position -1 with respect to the membrane-proximal Y⁷¹²SPL motif (Gly⁷¹¹) influences the recruitment of Env into clathrin-coated pits. Most importantly, these data establish a correlation between the binding of the cytosolic domain of Env to $\mu 2$, as well as to the intact AP2 complex, *in vitro* and the efficiency of Env internalization *in vivo*.

DISCUSSION

The intracellular domain of HIV-1 Env is absolutely required for viral dissemination, but very little is known about its functions. This prompted us to look for proteins interacting with the cytosolic domain of Env. Here we report that the medium chain $\mu 2$ of the clathrin-associated protein complex AP-2 as well as the complete AP-2 complex binds the cytosolic domain of Env fused to GST. Furthermore, Env and AP-2 form a complex *in vivo*, *i.e.* in infected lymphocytes. Binding of $\mu 2$ /AP-2 to the cytosolic domain of Env correlates well with Env internalization from the cell surface. Such fusion proteins may therefore be useful not only as probes to identify other proteins interacting with the cytosolic domain of Env but also to analyze the requirements of functional interactions of the adaptor protein complexes or its subunits with the cytosolic domain of Env.

Determinants in the cytosolic domain of Env have been implicated in the directed release of HIV-1 observed in polarized

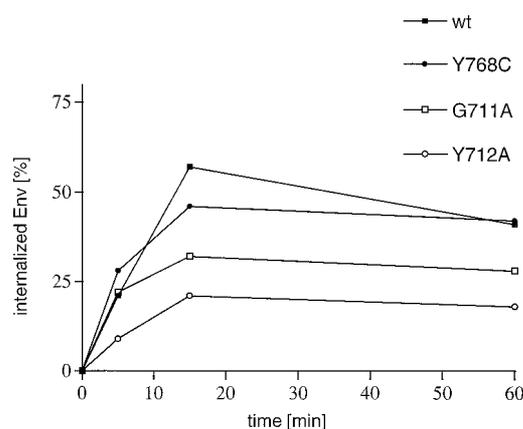


FIG. 6. Internalization kinetics of wild-type and mutant Env proteins. The amount of internalized Env was assessed using a biochemical endocytosis assay (see “Experimental Procedures”). Representative data from one of at least two experiments are shown.

epithelial cells as well as in the retrieval of Env from the cell surface (7, 8, 23, 24). In both instances, a membrane-proximal tyrosine (Tyr⁷¹²) was demonstrated to be part of the signal(s) responsible for the respective sorting process. Tyrosines have been identified as critical components of sorting signals in the cytosolic domains of different cellular membrane proteins destined for various cellular compartments (10, 12). Together, these data led us to investigate whether the cytosolic domain of Env interacts with elements of the cellular sorting machinery. As a probe, we used a protein where GST was fused to the cytosolic domain of Env. It was demonstrated previously that GST fusion proteins containing tyrosine-based sorting signals can bind proteins involved in the sorting of cellular membrane proteins (13). Here, we demonstrated that the cytosolic domain of Env in the context of a GST fusion protein binds to $\mu 2$. Such an interaction between the whole cytosolic domain of Env and $\mu 2$ was not observed in the yeast two-hybrid system,³ thus demonstrating the usefulness of the *in vitro* binding approach in order to study these interactions.

Having established that the cytosolic domain of Env in the context of a GST fusion protein can bind to $\mu 2$, we sought to define the requirements for this interaction. The cytosolic domain of Env contains two YXX Φ motifs. Both motifs are highly conserved among almost all primary viral isolates analyzed so far (18), indicating that they belong to functionally important regions of this domain. The results of the present study demonstrate that only the membrane-proximal tyrosine-based motif Y⁷¹²SPL, but not the more C-terminally located motif Y⁷⁶⁸HRL, is important for $\mu 2$ binding. The lack of a contribution of the YHRL motif to $\mu 2$ binding was not due to a lower intrinsic affinity of this motif for $\mu 2$. Using the yeast two-hybrid system, Ohno *et al.* (25) recently showed that the membrane-distal motif as such can bind to $\mu 2$. Our peptide competition experiments shown in Fig. 4 indicated that the YHRL motif may have even a slightly higher affinity for $\mu 2$. The results of the *in vitro* binding assays presented here thus suggest that $\mu 2$ cannot access the YHRL motif in the context of the whole cytosolic domain. Support for this hypothesis is provided by the analysis of Env internalization (Fig. 6). Our analysis as well as a previous study (7) found that tyrosine at position 712 but not tyrosine at position 768 is critical for Env internalization. Remarkably, the correlation between binding of $\mu 2$ to the cytosolic domain of Env and endocytosis of Env holds even for the Gly⁷¹¹ to Ala mutant. Glycine at position -1 relative to the critical Y⁷¹²SPL motif has not been previously recognized to

influence the internalization of membrane receptors. Mutant G711A showed an intermediate phenotype in the $\mu 2$ binding assay as well as in the endocytosis assay. Together, our data suggest that binding of $\mu 2$ to the cytosolic domain of Env defines the specificity for the recruitment of the protein by the AP-2 complex. Therefore, we tested whether the cytosolic domain of Env also associates with the intact AP-2 complex *in vitro* and, if so, whether the specificity of binding to EnvCD was the same for $\mu 2$ and the intact complex. Our results showed, first, that EnvCD-AP-2 association was likewise dependent on the intactness of the membrane-proximal but not of the membrane-distal tyrosine-based motif. Second, we could reproducibly precipitate the large chains α - and β -adaplin of AP-2 with the Gly⁷¹¹ to alanine mutant, although its interaction with the intact AP-2 complex may be weaker than its association with $\mu 2$. Therefore, the strength of interaction of $\mu 2$ with cognate signals in the cytosolic domains of membrane proteins may be modulated by an interaction of $\mu 2$ with the other subunits of AP-2.

Appropriate spacing of the tyrosine signal was shown to be crucial for the sorting of a cellular protein to lysosomes (26), presumably mediated by the AP-1 complex. A possible explanation for the different recognition by $\mu 2$ /AP-2 of the Y⁷¹²SPL motif and the Y⁷⁶⁸HRL motif may thus be due to different placement relative to the plasma membrane. Alternatively, folding of the cytosolic domain of Env may be such that Y⁷⁶⁸HRL is not accessible to $\mu 2$ /AP-2. The Y⁷⁶⁸HRL motif is positioned within one of two amphipathic regions of the cytosolic domain of Env that have been proposed to associate with the inner face of the plasma membrane (27, 28). It could thus be that the conformational context of the Y⁷⁶⁸HRL motif *in vivo* prevents its recognition by the AP-2 complex in cells. The situation may be different for the region close to the membrane-spanning domain of Env. This region is predicted to form neither α -helices nor β -sheets (29). The Y⁷¹²SPL motif within that context may thus adopt the “tight turn” structure, which was predicted to be the conformational determinant shared by tyrosine-based internalization signals (30). Our results do not allow us to exclude the possibility that the membrane-distal motif contributes to the EnvCD-AP-2 binding but that the interaction with Tyr⁷⁶⁸ is dependent on the interaction of AP-2 with the membrane-proximal motif. However, since the Tyr⁷⁶⁸ to Cys mutation does not affect Env internalization (Fig. 6), we do not consider this to be a very likely scenario.

Preliminary results suggest that the described Env fusion proteins not only bind with remarkable specificity to $\mu 2$ /AP-2 but that they can also selectively interact with other cellular sorting complexes. Work is in progress to test whether the association with those complexes also correlates with the *in vivo* sorting of Env. Such an analysis is particularly desirable with regard to the membrane-proximal region of the Env tail, which is involved not only in Env endocytosis (Ref. 7 and this study) but also in the polarized sorting of Env (24). This situation is reminiscent of the co-linearity of endocytosis signals and basolateral sorting signals that is observed in a number of cellular proteins (31).

In summary, these studies show that the $\mu 2$ subunit of AP-2 as well as the intact AP-2 complex bind to a tyrosine-based signal within cytosolic domain of Env involved in endocytosis. A second tyrosine-based motif in the cytosolic tail of Env that is not relevant for Env internalization seems to be largely shielded from recognition by $\mu 2$ /AP-2. In all, our data describe a case where components of the cellular sorting machinery discriminate between two potential binding sites based on their position in the cytosolic domain of the protein. Thus, our data demonstrate that it is not possible to predict if a putative $\mu 2$ binding sequence will function *in vivo*, based on *in vitro* studies

³ H. Ohno and J. Bonifacino, unpublished observations.

alone, underscoring the importance of corroborating data from binding experiments with functional studies *in vivo*.

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