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

Michael Boge‡, Stéphanie Wyss‡, Juan S. Bonifacino§, and Markus Thali¶

From the ‡Institute of Microbiology, University of Lausanne, CH-1011 Lausanne, Switzerland, and §Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

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, μ2, 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 (Y712SPL and Y768HRL), both of which are capable of binding to μ2 when presented as short peptides. However, only the membrane-proximal motif Y712SPL binds to μ2 and is required for internalization in the context of the whole cytosolic domain of Env. A glycine residue (Gly711) adjacent to the Y712SPL motif is also important for binding to μ2/AP-2 and internalization. These observations suggest that the accessibility of the membrane-proximal Y712SPL to μ2/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) 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). 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 β2), a medium chain (μ2), and a small chain (σ2). A direct interaction between μ2 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 μ2 chain of AP-2 as a protein that interacts with the cytosolic domain of Env. Binding of μ2 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 μ2 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 μ2 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 (Pcvo DNA polymerase; Boehringer Mannheim)-amplified DNA fragments into pGEX-3X (Amersham Pharmacia Biotech). Primer sequences were as follows: 5′-AATCCCCGAGTAGTTTTGCTGAC-3′ and 5′-CTTAAGACCATTGCGACCCCATCTTTA-3′. The polymerase chain reaction products were phosphorylated and inserted into SmaI-linearized pGEX-3X. The expression vectors 3M9 for μ2, 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. Sodorosi.

Site-directed mutagenesis was performed either in the GST fusion protein expression system or by PCR amplification of Env coding regions using primers either containing point mutations or containing the 5′ and 3′ primer sequences 5′-ACCGTACGCGATGCTGACCATCTTTA-3′ and 5′-TTCATGTCGAGATTGACACCGG-3′, respectively.

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Michael Boge‡, Stéphanie Wyss‡, Juan S. Bonifacino§, and Markus Thali¶

‡ 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.

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¶ To whom correspondence should be addressed: Institute of Microbiology, University of Lausanne, IMUL/CHUV 44, Rue du Bagnon, CH-1011 Lausanne, Switzerland. Tel.: 41 21 314 4099 Fax: 41 21 314 4095; E-mail: Markus.Thali@inst.hospvd.ch.

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constructs or in the Env expression vectors using the QuickChange™ system (Stratagene). The following primers were used: RT09A, 5'-GGTAATAGGCTGCGGGATATCC-3'; Q710A, 5'-GGTAAATAGGCTGCGGGATATCC-3'; G711A, 5'-GGTAAATAGGCTGCGGGATATCC-3'; Y712A, 5'-GGTAAATAGGCTGCGGGATATCC-3'; S713A, 5'-GGTAAATAGGCTGCGGGATATCC-3'; P714A, 5'-GGTAAATAGGCTGCGGGATATCC-3'; Y768C, 5'-GGTAAATAGGCTGCGGGATATCC-3'.

Binding of μ2/AP-2 to HIV-1 Env

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 μ2—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 (μ2) of the clathrin-associated protein complex AP-2 was found to interact with tyrosine-based signals conforming to the canonical motif YXXO, where X is any amino acid and O is an amino acid with a bulky hydrophobic side chain (13–15). Therefore, we decided

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-adaptin and β-adaptin, respectively). For comparison, an aliquot (1/50) of the T cell extract was loaded in lane 1.

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to test whether the cytosolic domain of Env of HIV-1 associates with μ2. The cytosolic tail was expressed as a GST fusion protein (GST-EnvCD) in E. coli. The medium chain (μ2) of the clathrin-associated protein complex AP-2 was translated in vitro in a rabbit reticulocyte lysate. Fig. 2 shows that μ2 bound to GST-EnvCD but not to GST. For comparison, one-tenth of the in vitro translated μ2 is shown in the first lane. Typically, about 5–15% of the in vitro translated μ2 bound to GST-EnvCD.

Binding of μ2 to the Cytosolic Domain of Env Depends on the Presence and the Context of a Tyrosine-based Motif—To identify the binding site for μ2 in the cytosolic domain of Env, we tested the interaction of μ2 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 μ2 with the cytosolic tail. Deletion of a region containing this membrane-proximal tyrosine-based motif, however, severely reduced the binding of μ2 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 μ2 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 μ2 to the cytosolic domain of Env. Similarly, substitutions of amino acids Arg709, Gln710, or Ser713, respectively, by alanine influenced binding of μ2 only marginally. In contrast, binding of μ2 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 μ2 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 μ2 to EnvCD. If protein-protein complexes were allowed to form in the presence of the peptide RRQGYSPL, binding of μ2 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. 2. Binding of the cytosolic domain of HIV-1 Env and μ2. In vitro translated [35S]Met-labeled μ2 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 μ2 to the immobilized recombinant proteins was analyzed on SDS-PAGE. For comparison, one-tenth of the in vitro produced μ2 was applied to lane 2.

Fig. 3. The membrane-proximal tyrosine-based signal is critical for the binding of μ2 to the cytosolic domain of HIV-1 Env. A, schematic drawing of different fusion constructs that were analyzed for μ2 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 μ2. Binding assays were performed as described in Fig. 2. C, binding of the different mutants to μ2. A representative polyacrylamide gel is shown. For quantitation, the amount of bound μ2 was analyzed as described under “Experimental Procedures.” The amount of μ2 bound to the mutants, expressed as the percentage of μ2 bound to the wild-type cytosolic domain of Env (±S.D.), was as follows: EnvCD, 100; R709A, 83.6 (±24.7); Q710A, 90.3 (±31.0); G711A, 14.2 (±2.7); Y712A, 6.0 (±6.5); S713A, 125.7 (±35); P714A, 41.3 (±9.2); L715A, 5.6 (±6); Y768A, 93.0 (±51); Y768C, 77.8 (±7.1). Data are from five experiments.
Determination of the cytosolic domain of Env important for functional interactions of the adaptor protein AP-2 with 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-adaptin, β-adaptin) 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.

Most interestingly, the peptide RRFSYHRL, the sequence of which conforms to the distal tyrosine-based signal, also interfered with the binding of μ2, 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 μ2 (see Fig. 3), it still was a potent μ2 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 μ2 Binding**—To test whether the same region of the cytosolic domain of Env important for μ2 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-adaptin, the Y712A mutant did not. Substitution of Gly711 for alanine, however, still allowed for the precipitation of some α- and β-adaptin. These results demonstrate that the association of not only the isolated medium chain μ2 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 μ2/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 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 μ2 and the intact AP-2 complex (Fig. 5) also reduced Env internalization. On the other hand, mutation of Tyr768, which had little effect on μ2/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 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 μ2, as well as to intact AP-2 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 μ2 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 μ2/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
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 μ2. Such an interaction between the whole cytosolic domain of Env and μ2 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 μ2, we sought to define the requirements for this interaction. The cytosolic domain of Env contains two YXXØ 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 Y768HRL, but not the more C-terminally located motif Y766HRL, is important for μ2 binding. The lack of a contribution of the YHRL motif to μ2 binding was not due to a lower intrinsic affinity of this motif for μ2. Using the yeast two-hybrid system, Ohno et al. (25) recently showed that the membrane-distal motif as such can bind to μ2. Our peptide competition experiments shown in Fig. 4 indicated that the YHRL motif may have even a slightly higher affinity for μ2. The results of the in vitro binding assays presented here thus suggest that μ2 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 μ2 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 μ2 binding assay as well as in the endocytosis assay. Together, our data suggest that binding of μ2 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 μ2 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-adaptin 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 μ2. Therefore, the strength of interaction of μ2 with cognate signals in the cytosolic domains of membrane proteins may be modulated by an interaction of μ2 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 μ2/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 μ2/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 μ2/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 μ2 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 μ2/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 μ2 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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