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Identification of the Cellular Prohibitin 1/Prohibitin 2 Heterodimer as an Interaction Partner of the C-Terminal Cytoplasmic Domain of the HIV-1 Glycoprotein▽

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Our studies aim to elucidate the functions carried out by the very long, and in its length highly conserved, C-terminal cytoplasmic domain (Env-CT) of the HIV-1 glycoprotein. Mass spectrometric analysis of cellular proteins bound to a tagged version of the HIV Env-CT led to the identification of the prohibitin 1 and 2 proteins (Phb1 and Phb2). These ubiquitously expressed proteins, which exist as stable heterodimers, have been shown to have multiple functions within cells and to localize to multiple cellular and extracellular compartments. The specificity of binding of the Phb1/Phb2 complex to the Env-CT was confirmed in various manners, including coimmunoprecipitation with authentic provirally encoded, full-length Env. Strong binding was dependent on Env residues 790 to 800 and could be severely inhibited by the double mutation L799R/L800Q but not by mutation of these amino acids individually. Analysis of the respective mutant virions revealed that their different abilities to bind Phb1/Phb2 correlated with their replicative properties. Thus, mutated virions with single mutations [HIV-Env-(L799R) and HIV-Env-(L800Q)] replicated similarly to wild-type HIV, but HIV-Env-(L799R/L800Q) virions, which cannot bind Phb1/Phb2, exhibited a cell-dependent replicative phenotype similar to that of HIV-Env-Tr712, lacking the entire Env-CT domain. Thus, replicative spread was achieved, although somewhat delayed, in "permissive" MT-4 cells but failed to occur in "nonpermissive" H9 T cells. These results point to binding of the Phb1/Phb2 complex to the Env-CT as being of importance for replicative spread in nonpermissive cells, possibly by modulating critical Phb-dependent cellular process(es).

In contrast to the glycoproteins of other membrane viruses, the cytoplasmic C-terminal domains (Env-CTs) of lentiviral glycoproteins are very long (in general over 100 amino acids [aa]). This feature is highly conserved *in vivo*, pointing to these regions carrying out essential and specific roles in lentiviral replication. The Env-CT of the human immunodeficiency virus type 1 (HIV-1) glycoprotein is 151 aa long, and mutant viruses with truncations within this region cannot replicate in most T-cell lines *in vitro* (14, 39). Such T-cell lines are referred to here as "nonpermissive" cells. However, in a few cell lines (notably MT-4 and C8166 T cells as well as in several commonly used adherent cell lines), mutant virions lacking the entire Env-CT are able to achieve replicative viral spread (33, 40, 51). These cell lines are referred to as "permissive" cell lines.

The HIV Env-CT exhibits a number of characteristic structural features and functional motifs. Within its C-terminal half there are two so-called lentiviral lytic peptide regions, namely, LLP1 (Env residues 826 to 854) and LLP2 (Env residues 768 to 788), as well as a leucine zipper-like motif (Env residues 793 to 814), which have been shown to interact with cellular membranes (8, 19, 28, 34). Due to these properties, and in analogy to proteins of several other viruses, it has been proposed that the HIV Env-CT domain may form a so-called viroporin with channel-like properties (11, 12). There are also several motifs within the Env-CT which interact with the cellular endocytotic machinery (44, 45, 55), and additionally, palmitylation of a cysteine residue is conserved in most HIV Env-CT domains.

There are genetic data (16, 39) and protein association data (10, 54) which indicate that, when present, the HIV Env-CT associates with the proximal matrix (MA) protein through an extended region spanning LLP2 (16). The functional consequence of this interaction is the inhibition of Env-mediated fusion in immature virus particles when MA is still part of the Gag precursor $Pr55^{gag}$ (38, 53, 54). The Env-CT, and in particular its interaction with MA, has also been implicated in involvement with incorporation of full-length viral glycoprotein into virion particles (16). Recently the cellular protein TIP47 has been proposed to play a role in this process by interacting with motifs within both the Env-CT and the MA protein (4, 32). However, although some shorter Env-CT truncations abrogate Env incorporation, Env protein lacking the entire Env-CT is incorporated into particles produced in "permissive" cells. On the other hand, in "nonpermissive" T cells in which mutant virions lacking the Env-CT cannot achieve replicative spread, it has been reported that incorporation of truncated Env into virions fails to occur (2, 40).

Most of the functional roles attributed to the Env-CT until now can also be fulfilled by nonlentiviral glycoproteins with

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much shorter CT regions (fewer than 50 aa). Thus, it seems likely that other specific functions of lentiviral Env-CTs have still to be identified. Elucidation of the identities of cellular proteins which interact with the Env-CT may give hints as to novel roles played by this region. Until now, in addition to interaction with proteins of the cellular endocytosis machinery, several reports have demonstrated that the Env-CT interacts with calmodulin (35, 50). Furthermore, using yeast two-hybrid screens, several further candidate proteins, including TIP47 as mentioned above, have already been identified. Thus, the Env-CT has been reported to interact with the guanine nucleotide exchange factor p115-RhoGEF and to inhibit p115-RhoGEF-induced actin stress fiber formation and serum response factor (SRF) activation (56). The prenylated Rab receptor (Rab1) and α-catenin are other reported interaction partners (15, 26, 27), and recently the Env-CT has been shown to interact with the cellular protein luman and to relieve its interference with Tat-mediated transcription of the HIV-1 long terminal repeat (LTR) (5).

The yeast two-hybrid screening technology, which has been employed for the detection of most of the currently identified Env-CT cellular interaction partners, is subject to inherent limitations. In particular, interactions with hetero-oligomeric protein complexes will not be identified. Thus, in the present study, we have employed affinity chromatographic and mass spectrometric (MS) procedures to identify proteins, endogenously expressed in human T cells, that are capable of interacting with a coexpressed tagged version of the Env-CT. In this manner, we have identified the dimer/oligomer consisting of the prohibitin 1 and 2 proteins as a novel interaction partner of the HIV-1 Env-CT. The Phb1 and -2 proteins, also known as B-cell receptor-associated proteins BAP32 and BAP37, respectively, are members of a superfamily including stomatin and flotillin (for a review, see reference 36). Prohibitin proteins have a similar domain topology (52) consisting of an N-terminal transmembrane domain (TMD), a structurally related prohibitin (PHB) domain which may facilitate partitioning into lipid microdomains (37), and a C-terminal coiled-coil domain. Phb1 and -2 exist as heterodimers, can form higher-structure oligomers (49), and not only are present in T cells but are ubiquitously expressed in tissues. They are predominantly found in mitochondria but also play roles in the nucleus, extracellularly in the circulation, and, of interest in the context of interaction with HIV Env, also at the cell membrane (36).

Here we describe the initial isolation of the Phb1/Phb2 proteins in association with the Env-CT, the data confirming their specific interaction with defined regions of the Env-CT, and experiments aimed to confirm a role for this interaction during HIV replication.

MATERIALS AND METHODS

Constructs. The construct pWPXLd-TAP-CT is an HIV-derived vector plasmid expressing the HIV Env-CT region N-terminally tagged with the so-called TAP tag (47). Sequences encoding the TAP tag were excised from plasmid pZome1N (Euroscarf, Frankfurt, Germany) with BamHI and EcoRI and inserted into pWPXLd (kindly provided by D. Trono, Geneva, Switzerland) from which the green fluorescent protein (GFP) gene had been excised with the same enzymes. The Env-CT (nucleotides 8370 to 8825, strain BH10 [20]) was amplified and inserted in frame 3′ of the TAP tag. pCMVΔR8.91 is a packaging plasmid for HIV-derived vectors (57), and pMD.G is an expression plasmid for the G glycoprotein of vesicular stomatitis virus (VSV-G) (41). The plasmid pWPI-Env-TMD-CT, expressing the Env-TMD-CT, i.e., the Env-CT anchored in the cellular membrane by the HIV Env membrane-spanning domain, has been described previously (22). The bacterial plasmid pMBP-Env-CT, expressing the maltose binding protein (MBP) fused with the Env-CT (i.e., Env aa 706 to 851) and derivatives thereof, were generated by in-frame insertion of the appropriate sequences into a modified pMAL plasmid (New England Biolabs). Mutated C-terminally truncated and internally deleted derivatives of pMBP-Env-CT (see Fig. 5 and 6) were generated using standard PCR procedures and their identities confirmed by sequencing. Control plasmids were either pMBP-HPV-L1 or pMBP-M2 expressing MBP fused to either the L1 protein of human papillomavirus type 16 or the influenza virus (strain Rostock) ion channel protein M2, respectively. pCMV-Phb1-FLAG and pCMV-Phb2-FLAG were kind gifts from H. Endo, Tochigi, Japan (25). pCMV-Phb2-FLAG mutants with deletions in the transmembrane (aa 2 to 36), prohibitin (aa 68 to 185), or coiled-coil (aa 190 to 264) domain (52) were generated by standard procedures and confirmed by sequencing.

The wild-type (WT) proviral construct pNL4-$3^{BH10env}$ (referred to here as pNL-WT) and mutant pNL-Env-Tr712, lacking 144 aa of the Env-CT, have been described previously (51). Mutated fragments leading to Env proteins with point mutations and C-terminal truncations within the Env-CT were generated by PCR and inserted into pNL-WT. Mutations were chosen such that the amino acid sequence of the Rev protein from the overlapping *rev* gene was not altered. The identities of all of these derivatives were confirmed by sequencing. The expression constructs for the subtype B Env clones, pRHPA4259 clone 7 and pTHRO4156 clone 18, and the Env-defective HIV particle expression plasmid, pSG3ΔEnv, have been described (30) and were obtained from the NIH AIDS Research and Reference Reagent Program.

Cells, transfections, and transductions. 293T cells were cultivated in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), and H9 and MT-4 T cells were cultivated in RPMI medium with 10% FCS. H9-GFP T cells have been described previously (22). 293T cells were transfected using either standard calcium phosphate procedures or with the deacylated polycation polyethylenimine (PEI87) as previously described (13). In order to generate HIV vector particles encoding TAP-Env-CT, 293T cells were cotransfected with pWPXLd-TAP-Env-CT, pCMVΔR8.91, and pMD.G. Released vector particles were concentrated 10-fold by ultrafiltration and employed to transduce 1 ml H9 T cells in the presence of 8 μg/ml Polybrene. This procedure was repeated once, and the resultant transduced cell population, referred to as H9-TAP-Env-CT cells, was expanded. Indirect immunofluorescence of the transduced cells employing nonimmune rabbit serum (NRS) showed that in comparison to untransduced H9 cells, all of the transduced H9 cells exhibited bright fluorescence as a result of binding of rabbit IgG to the protein A motifs of the expressed TAP-Env-CT (47). Western blot analysis showed the presence of a specific band at the correct position (calculated molecular mass, 36 kDa).

Affinity purification of TAP-tagged Env-CT. H9-TAP-Env-CT cells and H9-GFP cells, from 2.5 liters of the respective confluent cultures, were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and lysed in 0.1% Triton X-100, 100 mM NaCl, and 5 mM iodoacetamide in PBS containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After incubation for 15 min at room temperature, the lysates were clarified by centrifugation and the supernatants each incubated with 100 μl packed IgG-Sepharose (Amersham Biosciences, Uppsala, Sweden) at 4°C overnight with gentle agitation. The IgG-Sepharose samples were then washed 10 times on columns with 10 ml 0.1% Triton X-100 in PBS, and bound proteins were eluted with 1 ml 0.1 M glycine, pH 2.5. The eluates were immediately neutralized by addition of 100 μl 1 M Tris-HCl (pH 8.0) and concentrated almost to dryness on Mikrocon centrifugal filters (Millipore, Bedford, MA). The concentrated proteins were dissolved off the filters with approximately 50 μl 8 M urea and 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and approximately 5% of the total eluates was analyzed by polyacrylamide gel electrophoresis (PAGE).

Identification of proteins specifically bound to the Env-CT. Gels were fixed in 30% ethanol and 10% acetic acid overnight and protein bands stained using a nondestructive silver staining protocol essentially as previously described (48). Protein bands of interest were excised from the gel, cut into 1-mm pieces, repeatedly washed with water and 40 mM ammonium bicarbonate/ethanol (1:1, vol/vol), reduced with 10 mM dithiothreitol (DTT) at 56°C for 1 h, and alkylated with 55 mM iodoacetamide at 25°C for 30 min in the dark. After alkylation, gel plugs were repeatedly washed with 40 mM ammonium bicarbonate and ethanol, dehydrated with 100% acetonitrile, and air dried. Digestion with 10 ng/μl trypsin (Promega) was performed in 40 mM ammonium bicarbonate at 37°C overnight. For mass spectrometric identification, samples were prepared on AnchorChip 800/25 matrix-assisted laser desorption ionization (MALDI) target plates using

alpha-cyano-4-hydroxycinnamic acid as the matrix (17). MALDI–time-of-flight (MALDI-TOF) MS spectra were recorded in the positive-ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik Gmbh, Bremen, Germany). Ion acceleration was set to 26.5 kV, the reflector was set to 30.0 kV, and the first extraction plate was set to 20.6 kV. Mass spectra were obtained by averaging 50 to 350 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at *m/z* 842.50 and *m/z* 2211.10. Singly charged monoisotopic peptide masses were used as inputs for database searching. Searches were performed against the NCBInr database using the Mascot search algorithms (Matrix Science Ltd., United Kingdom). Isoelectric points were allowed to range from 0 to 14, carbamidomethylation was included as a fixed modification for cysteine side chains, and oxidation of methionine was allowed as a variable modification. Up to one missed tryptic cleavage was considered, and the mass tolerance for the monoisotopic peptide masses was set to ±75 ppm.

Coimmunoprecipitation assays with untagged Env-TMD-CT and full-length Env. For immunoprecipitation (IP) experiments, either approximately 1×10^7 293T cells were transfected with pWPI-Env-TMD-CT or 3×10^7 293T cells were transfected with pNL-WT or Env expression plasmids pRHPA4259 clone 7 and pTHRO4156 clone 18 plus the Env-defective HIV particle expression plasmid, pSG3ΔEnv. Detergent lysates (1% Triton) were prepared at 48 h posttransfection. In the case of TMD-Env-CT-expressing cells, lysates were made in 1% Triton in PBS and aliquots subjected to immunoprecipitation with gp41 monoclonal antibody (MAb) Chessie 8, which recognizes an epitope at the N terminus of the Env-CT (Env aa 727 to 732) (1). In the case of cells transfected with the proviral constructs, the entire cultures were lysed directly in 1 ml gp41 MAb hybridoma culture supernatant (approximately 10 μg specific IgG) containing 1% Triton X-100. After incubation overnight with protein G-Sepharose beads and thorough washing, immunoprecipitated TMD-CT was dissolved in gel electrophoresis sample buffer (SB). Immunoprecipitates from lysates of cells transfected with pNL derivatives were washed on a column and bound proteins eluted by two consecutive additions of 500 μl 0.1 M glycine, pH 2.5. The eluates were combined and immediately neutralized with 100 μl 1 M Tris-HCl (pH 8.0), concentrated on Mikrocon centrifugal filters, and finally taken up in SB. The immunoprecipitated materials were subjected to Western blot analysis with either anti-gp41 MAb, rabbit anti-gp120, or a mixture of antibodies to Phb1 and Phb2. These were originally purchased (anti-Phb1 from Santa Cruz Biotechnology, Santa Cruz, CA, and anti-BAP37 [Phb2] from BioLegend, San Diego, CA) but were subsequently generated by immunization of rabbits with purified glutathione *S*-transferase (GST)-Phb1 and GST-Phb2 proteins prepared in our laboratory. Comparative densitometric quantitation of specific bands on different exposures of the blots to film was carried out using the Image J software from the NIH.

Immunofluorescence of flagged Phb and HIV Env. COS-7 or HeLa cells were transfected with pCMV-Phb2-FLAG and the WT Env expression vector pLβAcenv (29). After 36 h, the cells were fixed in 3% paraformaldehyde and stained with FLAG M2 antibody (Sigma-Aldrich) and rabbit anti-gp120 serum.

Pulldown assays with MBP-Env-CT. The procedures employed for the expression of MBP fusion proteins in BL-21 bacteria, their recovery, and subsequent binding to amylose resin were carried out according to the manufacturer′s instructions (New England Biolabs). Untransfected 293T cells or 293T cells transfected with pCMV-Phb1-FLAG or pCMV-Phb2-FLAG were lysed with 1% Triton in PBS containing a cocktail of protease inhibitors (Roche, Mannheim, Germany), and aliquots of the clarified lysates were incubated at 4°C overnight with gentle agitation with aliquots of amylose resin which had bound equal amounts (as established by Coomassie blue staining) of the different fusion proteins. After washing with PBS, bound proteins were eluted with gel sample buffer. An aliquot of the eluates was subjected to PAGE followed by Coomassie blue staining (to visualize the amounts of fusion proteins employed), and a further aliquot was subjected to Western blot analysis with a mixture of antibodies to Phb1 and Phb2 or with FLAG M2 antibody.

Infection of T cells with mutant HIV carrying changes within the Env-CT. 293T cells were transfected with proviral plasmids encoding wild-type HIV or the respective mutated virions. At 48 h posttransfection, cell lysates were prepared (1% Triton in PBS with protease inhibitors) and virions in the culture supernatant were quantitated by HIV CA enzyme-linked immunosorbent assay (ELISA) (Innogenetics, Belgium). Aliquots of the cell lysates were subjected to Western blot analysis employing anti-gp120 serum, gp41 MAb Chessie 8, and p24 MAb (183-H12-5C) (9). Aliquots of the culture supernatants, each containing 100 ng CA, were added to 10^6 H9 or MT-4 T cells in 5 ml medium. At 5 hours postinfection, the infected cells were washed three times with medium and further cultivated. Aliquots of the infected cells and of the culture medium were collected daily and the infected cultures fed with fresh medium as required. Infected cells were dried on glass slides, fixed with methanol-acetone (1:1) at −20°C, and subjected to staining with human anti-HIV serum followed by fluorescently labeled anti-human IgG. The amounts of newly synthesized virions in the medium were quantitated by HIV CA ELISA (Innogenetics, Belgium). At late stages of infection (14 days postinfection [dpi]), further aliquots of the infected cells were collected and DNA extracted (QIAamp DNA Blood Mini kit; Qiagen, Hilden, Germany). DNA fragments spanning the mutations introduced in the Env-CT were amplified by PCR and subjected to sequencing.

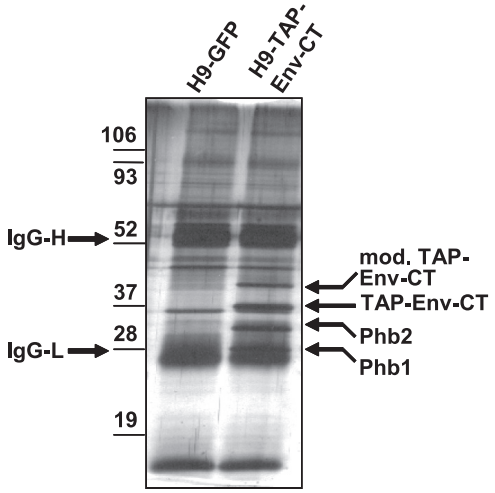

FIG. 1. Polyacrylamide electrophoresis and silver staining of proteins from H9-GFP cells or H9-TAP-Env-CT cells after affinity purification on IgG-Sepharose. IgG-H and IgG-L indicate the positions of the heavy and light chains of IgG, respectively, which have "bled" off the resin. Arrows indicate proteins specifically bound from the H9-TAP-Env-CT cell lysate and identified by MALDI-TOF.

RESULTS

Identification of Phb1 and Phb2 proteins specifically interacting with the HIV Env-CT. A tagged version of the HIV Env-CT (TAP-Env-CT, containing aa 706 to 851 of Env [see Materials and Methods]) was affinity purified from lysates of stably transduced H9 T cells (H9-TAP-Env-CT cells) and the identities of copurified cellular proteins established by mass spectrometry. Figure 1 shows gel electrophoresis of the proteins eluted from IgG-Sepharose after binding of lysates from H9-TAP-Env-CT cells or control H9-GFP cells. In addition to numerous protein bands seen in both preparations, four prominent bands were observed exclusively in the bound fraction from the H9-TAP-Env-CT cell lysate. The prominent diffuse bands, migrating at about 50 kDa and 25 kDa, seen in both preparations likely represent IgG heavy and light chains "bleeding" from the IgG-Sepharose. The four specific bands from the H9-TAP-Env-CT cell lysate were excised from the gel and subjected to tryptic digestion, and their identities were determined by MALDI-TOF. The band migrating at approximately 35 kDa was confirmed to be the TAP-Env-CT protein itself. Additionally, TAP-Env-CT sequences were detected in the slightly larger protein band migrating at about 42 kDa. The exact identity of this protein, which may represent a modified form of the TAP-Env-CT, has not been established. The proteins present in the two bands migrating at about 33 kDa and 28 kDa were identified to be the cellular proteins prohibitin 2

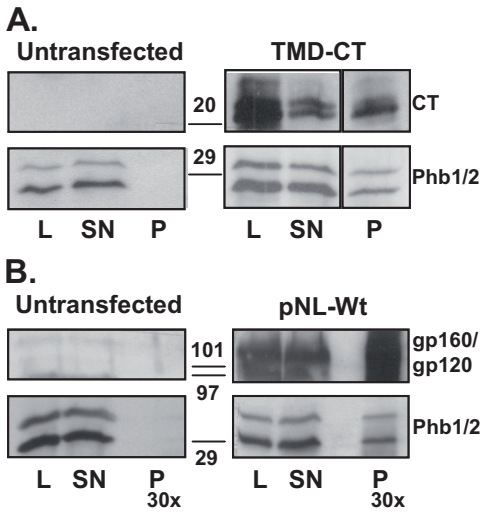

FIG. 2. Coimmunoprecipitation of Phb1/Phb2 with the Env-CT. (A) Lysates of untransfected cells or cells expressing a membrane-bound version of the Env-CT lacking the ectodomain (TMD-CT) were immunoprecipitated with gp41 MAb Chessie 8. Equivalent aliquots of the original lysate (L), of the supernatant remaining after immunoprecipitation (SN), and of the immunoprecipitated proteins (P) were probed in Western blotting with either Chessie 8 MAb (CT, top) or a mixture of antibodies against Phb1 and Phb2 (Phb1/2, bottom). (B) Lysates of untransfected cells or cells transfected with pNL-WT were immunoprecipitated with gp41 MAb Chessie 8. Immunoprecipitated proteins were concentrated 30-fold, and then equivalent aliquots of the original lysate (L), of the supernatant remaining after immunoprecipitation (SN), and of the concentrated immunoprecipitated proteins (P) were probed in Western blotting with either gp120 antibodies (gp160/gp120, top) or a mixture of Phb1 and Phb2 antibodies (Phb1/2, bottom).

(Phb2) and Phb1, respectively, which are known to form stable heterodimers (Phb1/Phb2) in cells.

Binding of Phb1/Phb2 to untagged Env-CT. In order to rule out that Phb1/Phb2 proteins were binding to the TAP tag present on TAP-Env-CT, untagged membrane-bound Env-CT (TMD-CT lacking the HIV Env ectodomain) expressed from pWPI-Env-TMD-CT and full-length Env expressed from pNL-WT were immunoprecipitated from transfected 293T cell lysates and examined for the presence of coimmunoprecipitated Phb1/Phb2. gp41 MAb Chessie 8, which recognizes an epitope within the CT, was employed in both cases. As shown in Fig. 2A, Phb1/Phb2 proteins were coimmunoprecipitated with the TMD-CT protein but were completely absent in the same IP from untransfected cells. In Fig. 2B, the IPs from lysates of cells expressing provirally encoded wild-type Env are shown. In this case, partially due to inefficient immunoprecipitation of full-length gp160 Env (and gp41 [not shown]), the immunoprecipitated sample was concentrated prior to analysis. It can clearly be seen that Phb1/Phb2 proteins were coimmunoprecipitated with WT Env but again were absent in the IP from untransfected cells. In order to rule out that interaction with Phb1/Phb2 is a property only of Env from HIV strain BH10 employed here, the same coimmunoprecipitation experiments were performed with two other Env proteins. The subtype B Env clones pRHPA4259 clone 7 and pTHRO4156 clone 18 (30) both contain the gp41 Chessie 8 epitope, required for immunoprecipitation, but otherwise differ significantly in the amino acid sequences of their cytoplasmic domains (at 24 and 16 Env-CT positions, respectively). However in both cases, Phb1/Phb2 proteins were coimmunoprecipitated to levels similar to those with pNL-WT encoding BH10 Env (data not shown). These experiments confirm genuine binding of Phb1/Phb2 proteins to untagged Env-CT.

Pulldown of Phb1/Phb2 proteins with immobilized MBP-Env-CT fusion proteins. In order to further confirm Phb1/Phb2 interaction with the Env-CT, maltose binding protein (MBP) fusion proteins with the Env-CT (MBP-Env-CT) were generated. Lysates of untransfected 293T cells, containing endogenously expressed Phb1 and Phb2, were incubated with MBP-Env-CT or MBP alone, immobilized on amylose resin. After this incubation and thorough washing, bound materials were probed in Western blotting for the presence of Phb1 and Phb2 proteins. As shown in Fig. 3A and B, pulldown of Phb1/Phb2 proteins from 293T cell lysates with MBP-Env-CT was efficient, while it was completely absent when only unfused control MBP protein was used. Protein bands at the positions of Phb1 and Phb2 were also absent when immobilized MBP-Env-CT was incubated in the absence of 293T cell lysate (not shown). Furthermore, Phb1 and Phb2 proteins did not bind to any of several other unrelated control fusion proteins. These were MBP fused to either the L1 capsid protein of human papilloma virus type 16 or the influenza virus protein membrane channel M2 (data not shown). In comparison to analysis of defined volumes of the cell lysate, we calculate that approximately 25% of the cellular Phb1/Phb2 proteins were pulled down by the MPB-Env-CT amylose resin.

In a further set of experiments, we examined whether exogenously expressed Phb1 or Phb2 proteins, and mutated derivatives thereof, also interacted with the Env-CT. For this, 293T cells were transfected with pCMV-Phb1-FLAG or pCMV-Phb2-FLAG, which encode Phb proteins C-terminally fused to three copies of the FLAG sequence. Figure 3C and D show Western blot analyses, employing FLAG antibodies, of the proteins from transfected cell lysates which have bound to immobilized MBP-Env-CT. FLAG-tagged Phb1, although expressed strongly, bound only weakly to MBP-Env-CT (Fig. 3C), whereas FLAG-tagged Phb2 interacted very efficiently and specifically (Fig. 3D). The reason for the failure of FLAG-tagged Phb1 to bind to the Env-CT is not known. In addition, a number of different derivatives of FLAG-tagged Phb2, lacking either the TMD, the prohibitin domain, or the coiled-coil domain, have been generated. None of these mutated derivatives were able to bind to the Env-CT (Fig. 3D), which indicates that binding to the Env-CT requires that Phb2 be present in an authentic conformation, presumably in a heterodimer with its endogenous partner Phb1.

Intracellular localization of Phb proteins and HIV-Env. Since the Env-CT interacts strongly with Phb1/Phb2 proteins, it was of interest to establish if Env and Phb proteins exhibit any cellular colocalization. In our hands, all available Phb antibodies gave rather weak and diffuse staining, which is why we rather opted to express FLAG-tagged Phb and employ anti-FLAG MAb to detect its localization. Based on the fact that FLAG-tagged Phb2, but not FLAG-tagged Phb1, interacted strongly with the Env-CT in pulldown assays (see above), we cotransfected pCMV-Phb2-FLAG and pLβAcenv, express-

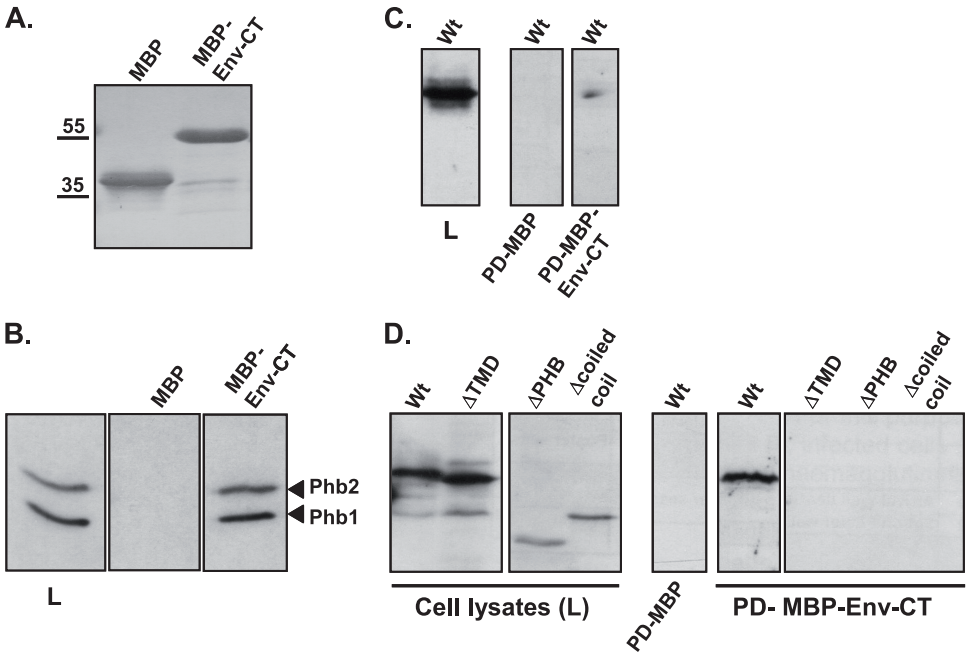

FIG. 3. Interaction of MBP-Env-CT fusion protein with endogenously and exogenously expressed Phb proteins. (A and B) Aliquots of 293T cell lysates were incubated with MBP or MBP-Env-CT, covalently bound to amylose resin. After washing, bound proteins were eluted in sample buffer, subjected to gel electrophoresis and analyzed either by Coomassie blue staining to establish the amounts of MBP and MBP-Env-CT proteins bound to the amylose resin (A) or in Western blotting employing a mixture of antibodies against Phb1 and Phb2 (pulldowns) (B). L, analysis of an aliquot (15% of the amount used for the pull-downs) of the original cell lysate. (C and D) Aliquots of lysates of cells expressing FLAG-tagged Phb1 (33 kDa) (C) or expressing FLAG-tagged Phb2 (36 kDa) or FLAG-tagged Phb2 derivatives lacking the transmembrane domain (ΔTMD, 32 kDa), the prohibitin domain (ΔPHB, 24 kDa), or the coiled-coil domain (Δcoiled coil, 28 kDa) (D) were incubated with MBP or MBP-Env-CT, covalently bound to amylose resin. Bound proteins were analyzed in Western blotting employing FLAG antibodies. L, analysis of an aliquot (25% of the amount used for the pulldowns) of the original cell lysate. PD, samples subjected to pulldown. All of the filters depicted in panels C and D were derived from a single experiment and exposure.

ing WT Env, into HeLa or COS-7 cells. These cells were chosen because of their large size and clear morphology. Since prolonged expression of exogenous Phb was cytotoxic (data not shown), transfected cells were fixed and stained at 36 h post-transfection. As shown in Fig. 4, FLAG-tagged Phb2 exhibits "spotty" staining, presumably reflecting localization in mitochondria, but additionally a more diffuse staining can be seen extending out to the cell periphery. HIV Env localizes mostly

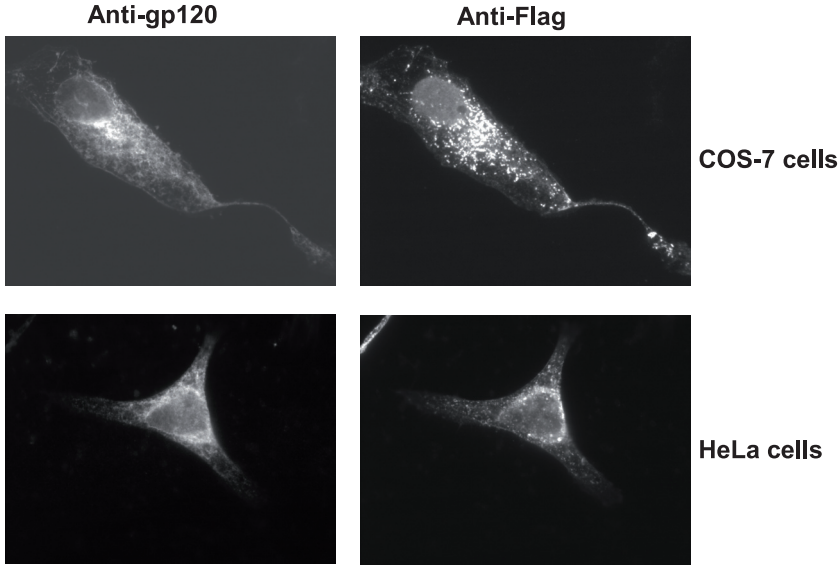

FIG. 4. Immunofluorescence of COS-7 or HeLa cells coexpressing HIV Env-WT and FLAG-tagged Phb2 with rabbit anti-gp120 or anti-FLAG MAb.

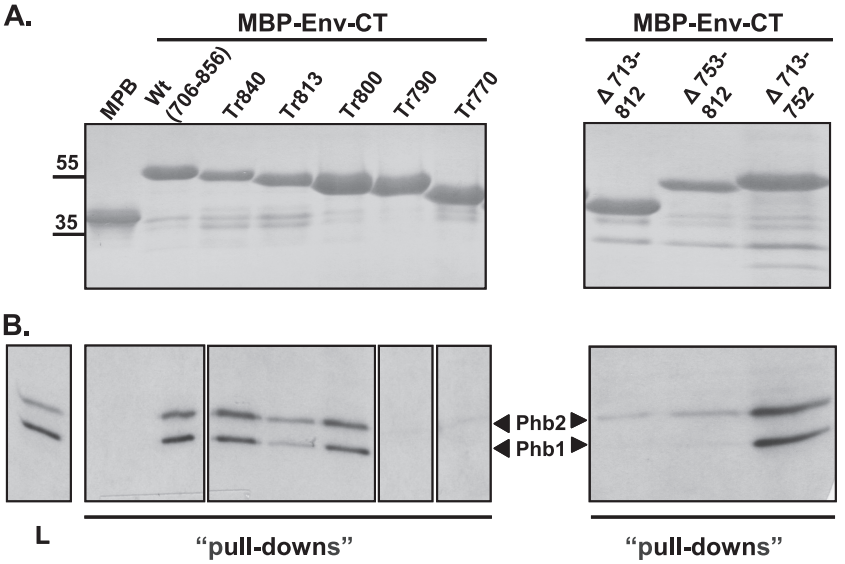

FIG. 5. Binding of Phb1/Phb2 to mutated derivatives of MBP-Env-CT. Aliquots of 293T cell lysates were incubated with either immobilized MBP, MBP-Env-CT, or C-terminally truncated or internally deleted derivatives of MBP-Env-CT as indicated. After washing, bound proteins were eluted in sample buffer, subjected to gel electrophoresis, and analyzed either by Coomassie blue staining (A) or by Western blotting employing a mixture of antibodies against Phb1 and Phb2 (pulldowns) (B). L, analysis of an aliquot (25% of the amount used for the pulldowns) of the original cell lysate. In panel B (left), all of the lanes were derived from a single experiment and exposure.

at a perinuclear location, presumably the endoplasmic reticulum and Golgi complex, but there is also weaker staining extending out to the cell periphery. Thus, although the individual staining patterns differ, there are clearly regions in the cell in which both Env and FLAG-tagged Phb2 are present.

Pinpointing the regions within the Env-CT important for Phb binding. In order to pinpoint regions important for binding, MBP-Env-CT fusion proteins containing several C-terminal truncations and internal deletions within the Env-CT domain were generated and analyzed for their ability to bind Phb1/Phb2 proteins. In the left panels of Fig. 5, a set of C-terminal truncation mutants of the Env-CT have been examined. Truncation of up to 56 aa from the C terminus (MBP-Env-CT-Tr800) did not reduce binding, but further truncations of 10 aa or 30 aa (MBP-Env-CT-Tr790 and MBP-Env-CT-Tr770) completely abolished Phb1/Phb2 binding. Thus, a region between aa 790 and aa 800 of Env appears to be critically involved in strong Phb1/Phb2 binding to the Env-CT.

In the right panels of Fig. 5, the abilities of MBP-Env-CT proteins with different internal deletions to interact with Phb1/Phb2 proteins have been examined. MBP-Env-CT Δ713–752, with a deletion of 40 aa at the membrane-proximal region of the Env-CT, bound Phb1/Phb2 as strongly as the WT. However, MBP-Env-CT Δ713–812, as well as MBP-Env-CT Δ753–812, exhibited greatly reduced binding. These proteins both lack a region between aa 752 and aa 812, again pointing to sequences within this region being important for strong Phb1/Phb2 binding. Nevertheless, weak binding of Phb1/Phb2 to both MBP-Env-CT Δ713–812 and MBP-Env-CT Δ753–812 could be seen and was reproducibly detected. Thus, there appears to be an additional, perhaps weaker, binding site for Phb1/Phb2 within the remaining regions of the Env-CT (i.e., the last 43 aa). Coomassie blue staining of parallel gels confirmed that, in all cases, approximately equal amounts of the respective fusion proteins had been bound to the amylose resin.

In summary, although the last 43 aa of the Env-CT in the absence of further sequences are capable of binding Phb1/Phb2 weakly, strong binding requires the presence of aa 790 to 800 of the Env-CT.

Point mutations in Env-CT sequences involved in Phb binding. With the aim of further narrowing down the critical amino acids in the Env-CT involved in Phb1/Phb2 binding, single and double amino acid exchanges within the region spanning aa 790 to 800 of Env were generated. In order to avoid confounding residual binding mediated by the C-terminal amino acids of the Env-CT region (as observed in MBP-Env-CT Δ713–812 [see above]), these small exchanges were made in the context of the MBP-Env-CT-Tr800 construct, which binds Phb1/Phb2 as strongly as full-length MBP-Env-CT. In Fig. 6A, the amino acid sequence of aa 788 to 800 of Env is shown, with highly conserved amino acids boxed and introduced amino acid changes indicated above and below. Two sets of mutant constructs were generated. In a first strategy, three tryptophan (W) residues within the region of interest were mutated to leucine. As shown in Fig. 6B, all of the MBP-Env-CT-Tr800 derivatives with single W-to-L mutations still strongly bound Phb1/Phb2.

In a second strategy, all of the amino acids between positions 788 and 800 of Env were exchanged pairwise to alanine (see sequence in Fig. 6A). As illustrated in Fig. 6C, mutation of R788A/G789A did not affect Phb binding at all. However all of the other exchanges significantly reduced but did not completely abolish binding. Of these, the W790A/E791A and L799A/L800A exchanges reduced Phb binding to the lowest level.

Proviral constructs with Env-CT mutations abrogating Phb binding. We were now interested in analyzing the phenotype of

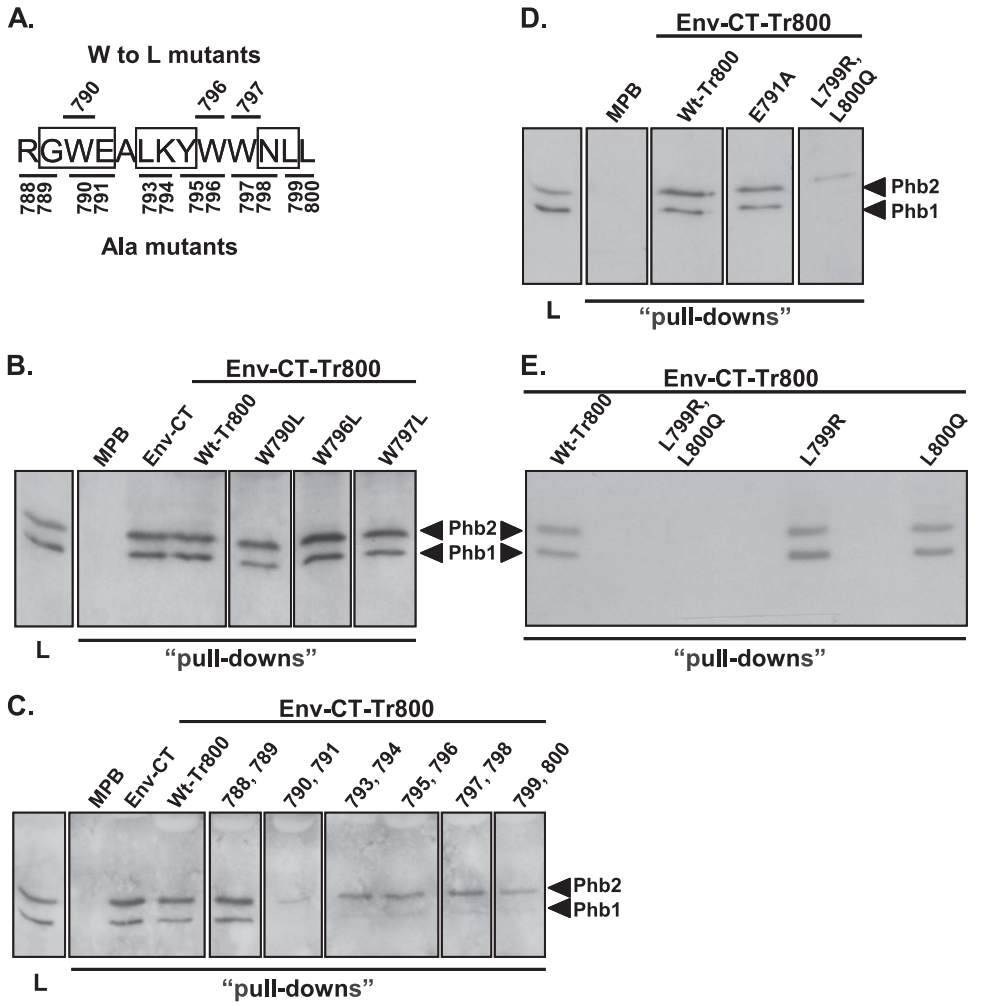

FIG. 6. Binding of Phb1/Phb2 to MBP-Env-CT-Tr800 mutated within Env aa 788 to 800. Aliquots of 293T cell lysates were incubated with either MBP, MBP-Env-CT, MBP-Env-CT-Tr800, or derivatives of MBP-Env-CT-Tr800 with single and double amino acid exchanges within the region from Env aa 788 to 800. Bound proteins were analyzed in Western blotting employing a mixture of Phb1 and Phb2 antibodies. (A) Sequence of Env residues 788 to 800, with conserved amino acids boxed. The positions of the W-to-L and the pairwise alanine exchanges, all generated in the context of MBP-Env-CT-Tr800, are shown above and below, respectively. (B) Analysis of the W-to-L exchanges. (C) Analysis of the pairwise alanine exchanges. (D to E) Analysis of the exchanges additionally examined in the proviral context. L, analysis of an aliquot (25% of the amount used for the pulldowns) of the original cell lysate. In each of panels B to D, all of the lanes were derived from a single experiment and exposure.

mutant virions encoding Env-CT domains with abrogated Phb1/Phb2 binding. Since the W790A/E791A and L799A/L800A exchanges had affected binding most strongly but, as shown in Fig. 6B, W790L had had no effect on Phb1/Phb2 binding, we focused on residues E791 and L799/L800. E791A and L799R/L800Q exchanges, which do not alter the overlapping *rev* gene, were generated in the proviral context as well as in the context of MBP-Env-CT-Tr800. As shown in Fig. 6D, MBP-Env-CT-Tr800, encoding the single amino acid exchange (E791A), still bound Phb1/Phb2 at the same level as the WT, and in fact, mutant virions encoding this exchange did not exhibit a defective phenotype (data not shown). However, similar to the case for L799A/L800A, the L799R/L800Q exchange did lead to a severe reduction in Phb1/Phb2 binding in the context of MBP-Env-CT-Tr800. This prompted us to additionally generate the singly mutated Env-(L799R) and Env-(L800Q) mutants. As shown in Fig. 6E, in the context of MBP-Env-CT-Tr800, these both regained the ability to bind Phb1/Phb2.

Infectivity phenotypes of WT and mutant virions with distinct Phb1/Phb2 binding capacities. Virions were generated in 293T cells transfected with pNL-WT, pNL-Env-(L799R/L800Q), pNL-Env-(L799R), and pNL-Env-(L800Q). Western blot analysis (Fig. 7A) of transfected cell lysates revealed that Gag (p24) and Env expression and processing were similar in all cases and, as determined by CA-ELISA, similar amounts of virions were released into the culture supernatants. The characteristics of the mutant virions with respect to productive viral spread were examined in permissive MT-4 or nonpermissive H9 T-cell lines. Equal amounts (as determined by HIV CA) of the respective virions and, as an informative control, of HIV-Env-Tr712 virions, encoding Env protein lacking the Env-CT (144-aa truncation), were employed. Input virions were removed at 5 h postinfection and the courses of the infections

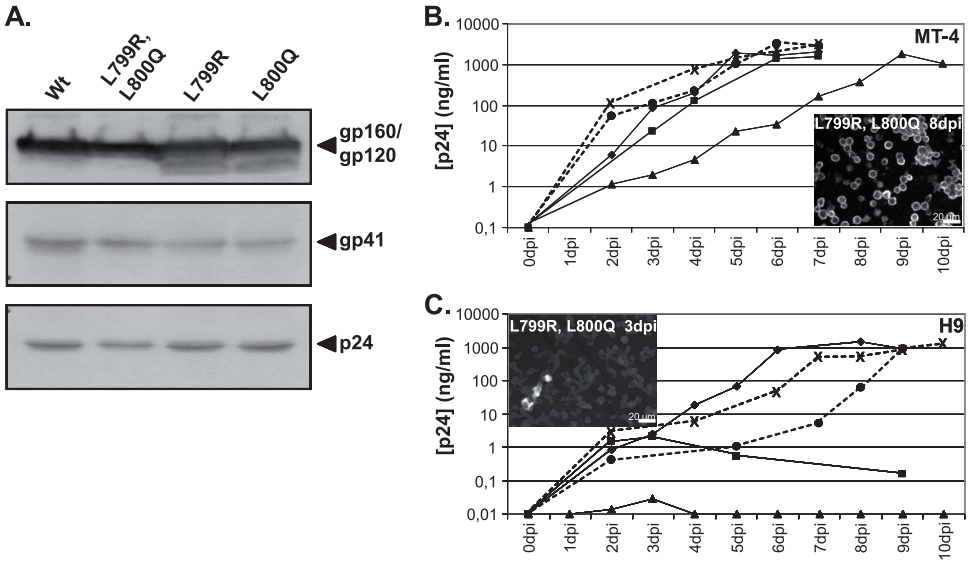

FIG. 7. Spreading infection of WT and mutant virions in MT-4 and H9 T cells. Virions were produced in 293T cells transfected with plasmid pNL-WT, pNL-Env-(L799R/L800Q), pNL-Env-(L799R), or pNL-Env-(L800Q) plasmids. (A) Western blot analysis of transfected 293T cells. The top part of the blot was probed with rabbit anti-gp120 serum, the middle part with gp41 MAb Chessie 8, and the bottom part with p24 MAb. (B) Replicative spread in "permissive" MT-4 cells of pNL-WT (solid line, diamonds), pNL-Env-Tr712 (solid line, squares), pNL-Env-(L799R/L800Q) (solid line, triangles), pNL-Env-(L799R) (dashed line, crosses), and pNL-Env-(L800Q) (dashed line, circles). The amounts of p24 released into the culture supernatants (as determined by p24 ELISA) are plotted against days postinfection (dpi). The inset shows immunofluorescence, determined using human anti-HIV serum, of cells infected with pNL-Env-(L799R/L800Q) at 8 dpi. (C) Replicative spread in "nonpermissive" H9 cells as described for panel B. The inset shows immunofluorescence, determined using human anti-HIV serum, of cells infected with pNL-Env-(L799R/L800Q) at 3 dpi. The cell density was approximately as in the inset in panel B, and the depicted field was chosen since positive cells were present.

analyzed by quantitation of the amounts of newly produced virions released into the culture supernatants over time. Additionally, infected cells were fixed at different time points and stained for HIV expression using human anti-HIV serum. As shown in Fig. 7B, in permissive MT-4 cells, WT HIV and all of the mutant virions were infectious. In the case of HIV-Env-(L799R/L800Q) virions, there was a delay in replication kinetics, but all of the cells in the culture did become infected (Fig. 7B, inset) and succumbed to HIV-induced cytotoxicity. Sequencing of integrated proviral HIV-Env-(L799R/L800Q) DNA confirmed that reversion to WT had not occurred. In contrast, in nonpermissive H9 cells, HIV-Env-(L799R/L800Q) and HIV-Env-Tr712 virions, which had been infectious in MT-4 cells, were unable to induce infectious viral spread (Fig. 7C). WT HIV and the two singly mutated variants HIV-Env-(L799R) and HIV-Env-(L800Q), which still retain Phb1/Phb2 binding, were able to replicate, albeit in the case of HIV-Env-(L799R) with delayed replication kinetics [again, sequencing of integrated HIV-Env-(L799R) DNA confirmed the presence of the mutated sequence]. Immunofluorescence revealed that initial infection by input virions (which had been generated in 293T cells) had been successful, and in all cases a small number of cells stained with HIV antibodies could clearly be seen (Fig. 7C, inset). These studies thus reveal that mutant HIV-Env-(L799R/L800Q), which is impaired in Phb1/Phb2 binding, exhibits a cell-specific infectivity phenotype resembling that of HIV-Env-Tr712 virions completely lacking the entire Env-CT. On the other hand, the singly mutated virions HIV-Env-(L799R) and HIV-Env-(L800Q), which have regained Phb1/Phb2 binding, have also regained the ability to replicatively spread in nonpermissive H9 cells. This suggests that Phb binding may be a determinant defining this phenotype and, as such, may be an important function of the Env-CT region.

DISCUSSION

Silver staining and MALDI-TOF analysis of cellular proteins bound to a tagged version of the HIV Env-CT led to the initial identification of prohibitin 1 and 2 proteins (Phb1 and Phb2) as potential interaction partners of the HIV Env-CT domain (Fig. 1). Phb1 and Phb2 are ubiquitously expressed membrane-bound proteins which belong to a larger family of proteins sharing a so-called stomatin/prohibitin/flotillin/HflK/C (SPFH) domain (7, 31, 37). They fulfill proposed roles in multiple aspects of cellular function, such as cellular signaling, transcriptional control, cellular senescence, apoptosis, and regulation of mitochondrial function (for a review, see reference 36). In congruence with this plethora of functions, Phb1 and Phb2 localize to multiple sites within the cell, whereby in many cells, the majority of Phb is present in mitochondria. However, Phb proteins have also been reported to be present in lipid raft domains of cellular membranes as well as in lipid droplets (6, 37).

The relative intensities of silver staining of the affinity-purified Phb1 and Phb2 protein bands were similar to one another and thus in line with the existence of the proteins as stable heterodimers in the cell (42). The amounts of bound Phb1 and Phb2 were estimated to be about half of that of the affinity-purified TAP-tagged Env-CT protein itself. However, in some subcellular compartments, in particular in mitochon-

dria, Phb proteins have been shown to form very large oligomeric structures (49), and thus multiple Phb1/Phb2 moieties may be associated with a single Env-CT domain. In addition to Phb1 and Phb2, there were no further detectable protein bands above the nonspecific negative-control background, which makes it likely that the interaction between Phb1/Phb2 and the Env-CT is direct.

Specific coimmunoprecipitation of endogenous Phb 1 and -2 proteins with coexpressed untagged, membrane-bound Env-CT (TMD-CT, lacking the Env ectodomain) or with full-length, provirally encoded functional Env protein ruled out a contribution of the TAP tag moiety to the initially detected interaction (Fig. 2). Phb1/Phb2 coimmunoprecipitation with TMD-Env-CT was very efficient. It was, however, less efficient, but completely specific, in the case of full-length Env (equivalent to approximately 3% of the total Phb1/Phb2), which may suggest that in correctly folded functional Env, the Phb1/Phb2 interaction site is less accessible. It is conceivable that Phb1/Phb2 binding to the Env-CT is restricted to a specific step in the HIV replication cycle and that, at steady state, only a fraction of the full-length Env protein is able to bind. Importantly, the Phb1/Phb2 interaction was not restricted to the Env-CT of the employed HIV strain BH10 but also occurred with two other, nonrelated Env proteins significantly differing in Env-CT sequence (in which, however, the LL799,800 residues were conserved).

Binding of endogenous or exogenous (FLAG-tagged) Phb1 and Phb2 to MBP-Env-CT, but not to any of a number of control MBP fusion proteins, again confirmed binding specificity. MBP fused with the M2 "viroporin" (ion channel) protein of influenza virus (18) was a particularly relevant negative control, since there is evidence suggesting that the HIV Env-CT domain may adopt a similar membrane-penetrating alpha-helical conformation as M2 (11, 12). Despite this putative similarity in structure, Phb1/Phb2 did not bind to MBP-M2 (data not shown). In the case of endogenous Phb1/Phb2 and FLAG-tagged Phb2, about 25% of the total cellular Phb bound to immobilized MBP-Env-CT. Specific binding of FLAG-tagged Phb1 to the Env-CT was, however, very much less efficient, which points to this tagged molecule being, at least partially, defective. Since Phb1 and Phb2 exist only as stable heterodimers in the cell, it is not possible to distinguish whether FLAG-tagged Phb1 is partially defective in binding to the Env-CT or whether it is unable to dimerize with its Phb2 partner. Mutated versions of FLAG-tagged Phb2 with deletions in the transmembrane domain, the prohibitin domain, or the coiled-coil domain, all completely failed to bind to the Env-CT (Fig. 3D). These data show that correctly folded and dimerized Phb1/Phb2 is a prerequisite for binding, but they do not reveal which protein moiety (Phb1 or Phb2) is the direct binding partner of the Env-CT. In further approaches to try to determine this, we have separately generated labeled Phb1 and Phb2 by *in vitro* transcription/translation as well as by use of bacterially expressed GST-tagged Phb1 and Phb2. However, none of these proteins alone or together were able to bind to immobilized MBP-Env-CT (data not shown), further indicating that only the authentic heterodimer can do so.

Immunofluorescence studies were performed to examine whether there were regions of cellular colocalization of FLAG-tagged Phb2 (which strongly binds to MBP-Env-CT) and WT Env. This is, of course, a prerequisite for these proteins to functionally interact in the cell. As mentioned above, cellular prohibitin proteins have been reported to perform manifold functions in different locations within cells. In HeLa or COS-7 cells (Fig. 4), FLAG-tagged Phb2 exhibited a predominantly "spotty" staining pattern, which is in line with its role as molecular chaperone in mitochondria (42, 43). However, additionally, more diffuse cytoplasmic FLAG-tagged Phb2 staining extending out to the cell periphery could be observed. As expected, HIV Env showed predominant staining in a perinuclear region but also weaker staining extending out to the cell periphery. Thus, although the individual staining patterns were different, there were clearly regions of costaining of both proteins, although at this level of sensitivity, it is not possible to say if this represents true colocalization or not.

Extensive small interfering RNA (siRNA) experiments to knock down Phb1/Phb2 expression in several different cell lines (and most importantly in susceptible T-cell lines) were performed with the aim of establishing the putative role of the interaction of Phb1/Phb2 with the Env-CT in HIV replication and spread. Unfortunately, these attempts were unsuccessful, since reduction of cellular Phb1/Phb2 amounts were cytotoxic within 36 to 48 h posttransfection of the siRNA constructs (not shown), and this precluded analysis of the viral phenotype. As an alternative, we thus sought to generate mutant HIV virions defective in Phb1/Phb2 binding to the Env-CT and to compare their phenotype with respect to productive viral replication and spread to that of pNL-WT and to mutant pNL-Tr712, which completely lacks the Env-CT domain and thus is defective in all Env-CT functions.

Initial examination of Phb1/Phb2 binding to MBP-Env-CT fusion proteins with truncations and large deletions within the Env-CT (Fig. 5) revealed that strong Phb1/Phb2 binding involves residues 790 to 800 of Env. However, residual Phb1/Phb2 binding to MBP-Env-CT Δ753–812, containing only the last 43 Env residues, pointed to there being a further Phb1/Phb2 interaction site in this region. Further studies of individual Env residues within the region from aa 790 to 800 revealed that the double mutation of L799/L800 either to AA or to RQ (the latter does not alter the overlapping *rev* gene) almost completely abrogated Phb1/Phb2 binding to MBP-Env-CT-Tr800. On the other hand, individual mutation of these residues (i.e., L799R and L800Q) had no detectable effect. Analysis of the respective mutant virions revealed that these different abilities to bind Phb1/Phb2 correlated with replicative properties in permissive MT-4 and nonpermissive H9 cells. Thus, in MT-4 cells, replicative spread of pNL-Env-(L799R/L800Q) did occur and all of the cells became infected, albeit in a delayed fashion. On the other hand, similar to the case for pNL-Env-Tr712, replicative viral spread of pNL-Env-(L799R/L800Q) in H9 cells was completely inhibited. In other words, the altered (inhibited) Phb1/Phb2 binding properties of pNL-Env-(L799R/L800Q) had an effect on mutant virus replication in cell culture similar to that of complete removal of the Env-CT domain. In contrast to this, pNL-Env-(L799R) and pNL-Env-(L800Q) virions, which had recovered Phb1/Phb2 binding, were able to replicate in both MT-4 and H9 cells. This suggests that authentic Phb1/Phb2 binding may be an important feature of the Env-CT and may be essential for viral spread in nonpermissive cells.

We have been unable to detect any differences in localization and gross trafficking between WT Env and the mutant Env constructs analyzed here. Proteolytic processing was similar in all cases (Fig. 7A), and the immunofluorescence staining patterns (of both Env and coexpressed FLAG-tagged Phb2 proteins) in cells expressing the WT or any of the mutant constructs could not be distinguished (data not shown). Furthermore, using procedures described previously (21, 23), we have established that incorporation of Env (gp120 and gp41) into pNL-WT and pNL-Env-(L799R/L800Q) virions, produced in nonpermissive H9 cells, was similar (data not shown). This indicates that the defective phenotype of pNL-Env-(L799R/L800Q) in nonpermissive cells cannot be attributed to an Env incorporation defect. This has been reported to be the underlying deficiency of pNL-Env-Tr712 (2, 40), an issue which is, however, controversial (21, 23).

The L799/L800 residues lie within the putative leucine zipper domain (aa 793 to 814) of the Env-CT, with L800 being the second leucine within the motif $LX_6LX_6LX_6L$. Previous studies have implicated this region as being involved in interacting with several other cellular proteins. Thus, the L800 residue has been reported to be important for binding of p115RhoGEF to the Env-CT (56), and mutant virions in which this residue has been replaced by arginine exhibit a defective phenotype in some cell lines (including H9 cells) and *in vivo* in SCID-hu Thy/Liv mice (24). However, in the present study, the L800Q mutation did not markedly affect replication kinetics in H9 cells (Fig. 7C). This indicates that the exact nature of the amino acid exchange at Env position 800 may be critical and that the presence of leucine is not mandatory for Env-CT function. It has further been reported that binding of α-catenin (27) as well as of the prenylated Rab receptor (PRA1) (15) to the Env-CT involves the central leucine-rich domain and that residues Y802 and W803 are involved in binding of TIP47 (4, 32). It is thus possible that, in addition to being defective in Phb1/Phb2 binding, pNL-Env-(L799R/L800Q) virions may be defective in other functions involving the putative Env-CT leucine zipper domain and that these additional phenotypes may contribute to the defective replication phenotype in nonpermissive cells. We cannot strictly rule out this possibility. However, as discussed above, the fact that the singly mutated constructs (L799R and L800Q) are able to replicate in nonpermissive H9 cells shows that these residues, at least singly, are not essential for further Env-CT function and replication.

We can only speculate as to the putative functional role of Phb1/Phb2 binding to the Env-CT during HIV replication and as to why, when binding is defective, replication is specifically abrogated in nonpermissive cells. Western blot analysis showed that there was no detectable difference in Phb1/Phb2 content in permissive MT-4 cells and nonpermissive H9 cells (data not shown), and analyses of their localizations/properties in these different cell lines have not been successfully performed to date. Depending on the cell type, prohibitin proteins have been reported to fulfill multiple functions at different subcellular locations. Thus, they serve major functions in mitochondria (for a review, see reference 3), where they assemble into ring-like macromolecular structures at the inner mitochondrial membrane and have been implicated to serve as chaperones for imported proteins. On the other hand, within the nucleus, both Phb1 and Phb2 interact with a variety of nuclear proteins involved in gene transcription and have been implicated as modulators of gene expression (for a review, see reference 36). Considering the presently known localization of the Env-CT at the cytoplasmic face of intracellular and surface membranes, it is difficult to envisage that HIV infection would affect prohibitin mitochondrial or nuclear functions. However, Phb1 and Phb2 proteins have additionally been identified on the plasma membrane, in lipid droplets, and in lipid raft preparations (for a review, see reference 36), and Phb1 has, for example, been shown to play a role in regulating signal transduction involving cRaf (46). At these cellular sites, binding of the Env-CT to Phb1/Phb2 may occur and as a result may locally influence (either positively or negatively) a yet-unidentified normal function of Phb1/Phb2 in a manner which is essential for HIV replication in "nonpermissive" T cells. Future investigations will aim at shedding light on these issues.

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