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A Novel Human WD Protein, h-βTrCP, that Interacts with HIV-1 Vpu Connects CD4 to the ER Degradation Pathway through an F-Box Motif

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Summary

HIV-1 Vpu interacts with CD4 in the endoplasmic reticulum and triggers CD4 degradation, presumably by proteasomes. Human βTrCP identified by interaction with Vpu connects CD4 to this proteolytic machinery, and CD4–Vpu–βTrCP ternary complexes have been detected by coimmunoprecipitation. βTrCP binding to Vpu and its recruitment to membranes require two phosphoserine residues in Vpu essential for CD4 degradation. In βTrCP, WD repeats at the C terminus mediate binding to Vpu, and an F box near the N terminus is involved in interaction with Skp1p, a targeting factor for ubiquitin-mediated proteolysis. An F-box deletion mutant of βTrCP had a dominant-negative effect on Vpu-mediated CD4 degradation. These data suggest that βTrCP and Skp1p represent components of a novel ER-associated protein degradation pathway that mediates CD4 proteolysis.

Introduction

The ability of viruses to subvert cellular functions to promote their own replication has often been used as a model to study general cellular pathways, including that of ubiquitin-mediated degradation of proteins. In most cases, proteins are targeted for degradation by the multicatalytic proteasome complex following covalent attachment of ubiquitin molecules mediated by ubiquitin-conjugating enzymes known as E2 after receiving activated ubiquitin from the upstream ubiquitin-activating enzyme E1 (for review, see Ciechanover, 1994). E2s can directly transfer ubiquitin to substrates, in concert with E3 ubiquitin-protein ligases. E3 activities provided by diverse protein complexes are believed to be largely responsible for the specificity of the ubiquitin-conjugation system (Weissman, 1997). For instance, in the control of the cell cycle, Sic1p, a B-type cyclin kinase inhibitor, is targeted for degradation at the G1-S transition (King et al, 1996; Willems et al., 1996) by a complex including Cdc4p/Skp1p. Cdc4p contains WD repeats and an F-box motif that mediates the connection to the ubiquitin-proteasome machinery by interacting with Skp1p (Zhang et al., 1995; Bai et al., 1996; Connelly and Hieter, 1996). Degradation of the tumor supressor factor p53 in papillomavirus-infected cells provides yet another example demonstrating how specificity of the ubiquitin-proteasome machinery can be conferred indirectly by viral proteins (Scheffner et al 1993).

The human immunodeficiency virus type-1 (HIV-1) Vpu protein might similarly act as an adapter molecule that links ER-associated CD4 to the ubiquitin-proteasome pathway. Vpu is an 81 amino acid integral membrane phosphoprotein expressed by most isolates of HIV-1, but not HIV-2 or simian immunodeficiency virus (Cohen et al., 1988; Strebel et al., 1988). Vpu mediates degradation of CD4, the major cellular receptor for HIV-1 (Willey et al., 1992), and contributes to the decrease in the expression of major histocompatibility complex (MHC) class I molecules on the surface of HIV-1 infected cells (Kerkau et al., 1997). Recent studies demonstrated that Vpu-mediated degradation of CD4 is sensitive to proteasome-specific inhibitors and is dependent on the presence of an intact ubiquitination machinery (Fujita et al., 1997; Schubert et al., 1998).

Down-regulation of viral receptors is a common mechanism of superinfection interference employed by a majority of retroviruses (reviewed by Bour et al., 1995a). However, HIV-1 is unique in its ability to interfere with cell-surface CD4 expression at three distinct levels (Chen et al., 1996). Early in the infection, the viral Nef protein down-regulates CD4 by accelerating its internalization and subsequent degradation in lysosomes (reviewed by Trono, 1995). At later stages of infection, CD4 retained in the endoplasmic reticulum with the envelope precursor gp160 (Crise et al., 1990; Bour et al., 1991) is targeted to degradation by Vpu (Willey et al., 1992). Vpu has therefore a unique role in that it not only contributes to the overall down-regulation of cell-surface CD4 but also allows the envelope precursor to be released into its normal transport and maturation pathway.

Available evidence suggests that CD4 degradation is a multistep process that is initiated by a physical interaction with Vpu (Lenburg and Landau, 1993; Vincent et al., 1993; Bour et al., 1995b; Margottin et al., 1996). Two conserved phosphoserine residues at positions 52 and 56 in the cytoplasmic domain of Vpu are required to trigger CD4 degradation (Schubert and Strebel, 1994; Friborg et al., 1995). However, they are not required for the initial binding to CD4 (Bour et al., 1995b). The sensitivity of CD4 degradation to proteasome inhibitors suggests the involvement of cytosolic proteasomes in this process. However, it is unclear how CD4, which is a transmembrane protein with only a short cytoplasmic component, is targeted to the proteasome machinery. ER-associated degradation (ERAD) of misfolded or otherwise damaged proteins can proceed through the cytosolic ubiquitin-proteasome pathway (Werner et al.,

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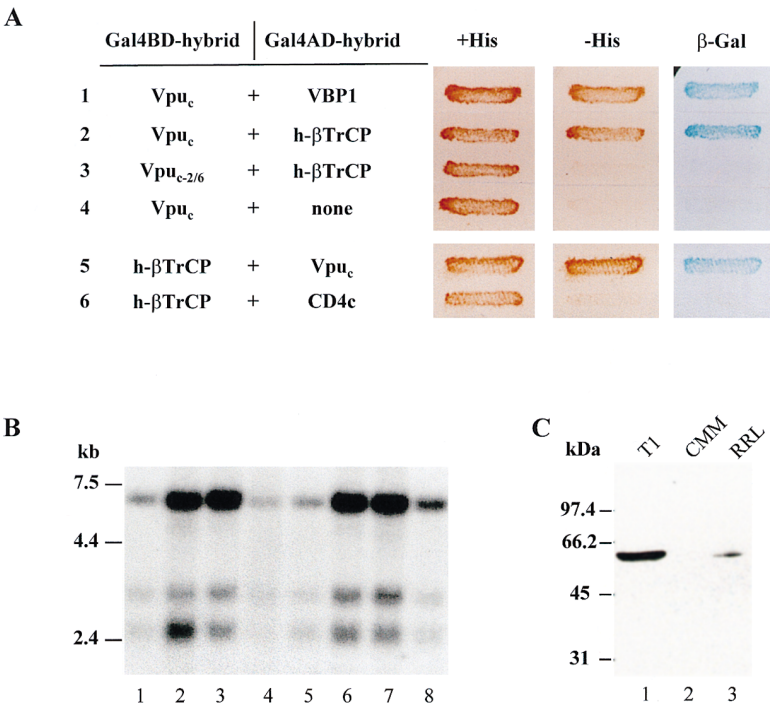

Figure 1. Characterization of Human βTrCP

(A) Specific interaction of the cytoplasmic domain of HIV-1_{Lai} Vpu (Vpu$_c$) with h-βTrCP in the two-hybrid system. The yeast reporter strain HF7c expressing the pairs of indicated hybrid proteins was analyzed for histidine auxotrophy and β-galactosidase expression. Transformants were plated on medium with histidine (left, lanes 1–6), or without histidine in the presence (middle, lanes 1–4) or in the absence (middle, lanes 5 and 6) of 10 mM 3-amino-1,2,4-aminotriazole (3AT), or replica-plated on Whatman filters and tested for β-galactosidase activity (right). Growth in the absence of histidine and blue color in the β-galactosidase activity assay indicate interaction between hybrid proteins.

(B) h-βTrCP mRNA expression. hβTrCP mRNA expression was analyzed in multiple human cell lines by Northern blot (from Clonetech), using a probe corresponding to a 5′ HindIII fragment of h-βTrCP cDNA. Size of RNA markers is indicated on the left. Poly(A)$^+$ RNAs were from the following cell lines: HL60, Hela S3, K562, Molt-4, Raji, SW480, A549, and G361 (lanes 1–8, respectively).

(C) h-βTrCP protein expression. h-βTrCP protein expression was analyzed by Western blot with anti-h-βTrCP anti-peptide 275–293 antibodies in lysates from human SupT1 T cell line (T1) (lane 1), canine microsomal membranes (CMM) (lane 2), and rabbit reticulocytes (RRL) (lane 3).

1996). Proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) with large cytoplasmic domains protruding in the cytosol can presumably be directly targeted by membrane-associated E2 activities (Jensen et al., 1995; Ward et al., 1995). Luminal proteins or proteins lacking the lysine residues required for ubiquitin conjugation on their cytoplasmic tails may be removed from the ER for degradation in the cytosol (Hiller et al., 1996). Such a mechanism was recently described for human cytomegalovirus (HCMV) US2 and US11 proteins that mediate the cytosolic export and subsequent degradation by the proteasome machinery of ER-associated MHC class I molecules (Wiertz et al., 1996).

The objective of the present study is to investigate the mechanism of Vpu-mediated ER degradation of CD4 and to identify the cellular proteins involved. We report the isolation of a novel human protein, h-βTrCP, which specifically associates with Vpu. Human βTrCP is a member of the WD proteins family that displays strong homology with the *Xenopus laevis* βTransducin repeats-containing protein (x-βTrCP) (Spevak et al., 1993). h-βTrCP also contains an F-Box motif that interacts with Skp1p. These results suggest that h-βTrCP and Skp1p are members of a novel ERAD pathway that mediates Vpu-dependent proteolysis of CD4.

Results

Human βTrCP Is a Vpu Binding Protein

Taking advantage of the yeast two-hybrid system we identified a cellular protein that directly interacts with Vpu and could be implicated in its function. The cytoplasmic domain of Vpu (Vpu$_c$), which is known to carry signals required for CD4 degradation, was chosen as a bait. Vpu amino acid residues 28–81 of the HIV-1 Lai isolate were fused to the Gal4 DNA binding domain (Gal4BD). A Jurkat cells cDNA library fused to the Gal4 activation domain (Gal4AD) was screened (Benichou et al., 1994). A 1.3 kb cDNA encoding VBP1 (Vpu Binding Protein 1), a polypeptide of 319 amino acid residues that specifically interacted with Vpu$_c$ (Figure 1A, lane 1), was isolated. VBP1 is highly similar (over 98% identity) to the C-terminal sequence of x-βTrCP1 (Spevak et al., 1993) and is thus likely to represent part of the human homolog of x-βTrCP1 (h-βTrCP). A 2.1 kb cDNA with the complete coding sequence for h-βTrCP was subsequently isolated by PCR (Figure 2). Interaction of Vpu$_c$ with full-length h-βTrCP was comparable to that obtained with VBP1 (Figure 1A, lanes 1 and 2) and was detected irrespective of whether Vpu$_c$ was fused to the Gal4BD or the Gal4AD (lanes 2 and 5). Interaction was not detected with an empty vector (lane 4) or with the cytoplasmic domain of CD4 (CD4c) (lane 6).

The h-βTrCP ORF comprises 569 amino acid residues. Comparison with the x-βTrCP sequence revealed a striking similarity of the two proteins up to 88% amino acid identity, with only 16 amino acid differences, mainly at the N and C termini, indicating that this protein is highly conserved from *X. laevis* to human (Figure 2). Hudson and colleagues (1996) described a partial cDNA clone coding for x-βTrCP2 isoform which has an extension of 16 residues at the C terminus highly homologous to that found in h-βTrCP (not shown). Three βTrCP transcripts of 2.5, 3.5, or 4.9 Kb length have been recently detected

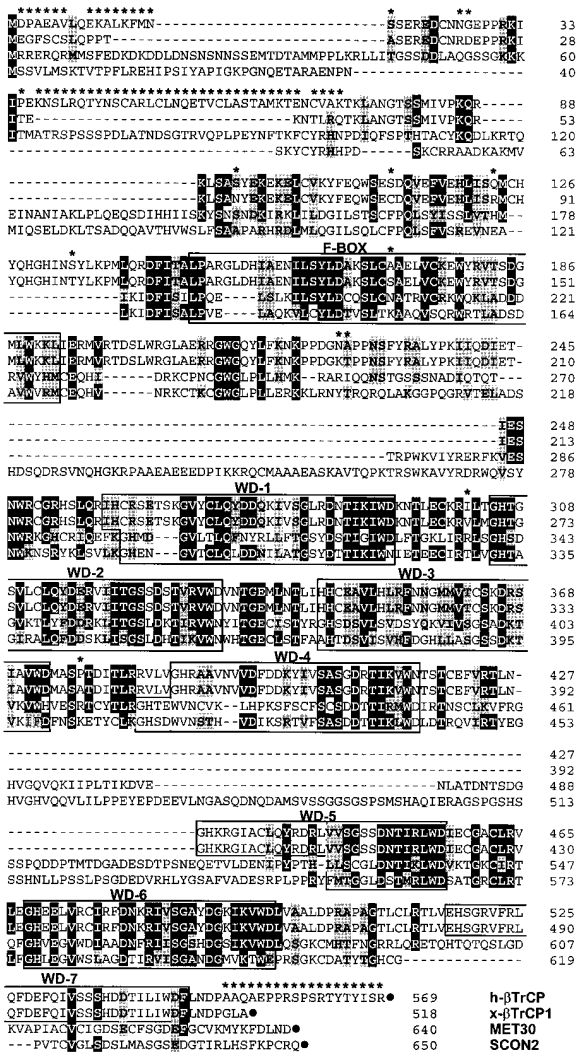

Figure 2. Alignment of Human βTrCP Sequence with *Xenopus* βTrCP1, *S. cerevisiae* Met30p, and *Neurospora crassa* Scon2p Sequences

Positions with at least three amino acid identities are shown as closed boxes, and positions with at least three conservative changes in shaded boxes. The F-box motif and the seven WD repeats are indicated. Stars above the h-βTrCP sequence indicate the differences between h-βTrCP and x-βTrCP1 sequences. The h-βTrCP accession number is Y14153.

in *Xenopus* (Hudson et al., 1996). Similarly, Northern blot analysis of RNAs from multiple human cell lines using either a 5′ or a 3′ probe revealed three h-βTrCP transcripts, a major one of 7 kb and two minor ones of 2.5 and 3.5 kb expressed in all cell lines tested (see Figure 1B).

In Western blot analysis, antibodies raised against a peptide encompassing residues 275–293 of h-βTrCP allowed us to detect a single protein band of approximately 60 kDa in lysates of the human T lymphocytic Sup T1 cell line (Figure 1C, lane 1), or in rabbit reticulocyte lysates (presumably rabbit βTrCP) (lane 3), but not in canine microsomal membranes (CMM) (lane 2). The size of this band correlates well with the size predicted from the h-βTrCP ORF (Figure 2).

Human βTrCP Contains an F-Box Motif and Seven WD Repeats

Two major domains can be distinguished in h-βTrCP (see Figure 2). The N-terminal domain contains an F-box motif (residues 148–192) recently identified as a sequence involved in the targeting of proteins to the ubiquitin-mediated proteolytic pathway, through interaction with Skp1p (Bai et al., 1996). The C-terminal domain of h-βTrCP contains seven WD repeats, originally identified in β subunits of heterotrimeric G proteins and known to form interfaces for protein–protein interactions (Neer et al., 1994). The seven WD repeats of h-βTrCP are identical to those of x-βTrCP1, and only one amino acid difference is found in the F-box region of the two proteins (see Figure 2).

Data-base search for homologous proteins revealed that *Saccharomyces cerevisiae* Met30p, a transcriptional inhibitor involved in the biosynthesis of sulfur amino acids (Thomas et al., 1995), as well as Met30p functional homologs such as Scon2p, are among the closest relatives of h-βTrCP. Another protein related to h-βTrCP but with lower degree of similarity is Cdc4p (Yochem and Byers, 1987). Alignment of h-βTrCP, x-βTrCP1, Met30p, and Scon2p is shown in Figure 2. The homology found between these proteins and βTrCP is overall limited, reaching 33% and 31% similarity for Met30p and Scon2p, respectively. This suggests that there is no true homolog of βTrCP in yeast. Indeed, h-βTrCP was not capable of complementing Met30p deficiency in *S. cerevisiae* (data not shown).

The WD Repeats Are the Binding Site for Vpu in h-βTrCP

Results of the initial two-hybrid screen showed that the C-terminal fragment of h-βTrCP with the seven WD repeats was sufficient for binding to Vpu (Figure 1, lane 1). Deletion of the first WD domain (VBP1-ΔW_1) abolished the interaction with Vpu (Table 1). In contrast, a weak interaction was still detected with mutants lacking either the C-terminal tail after the seventh WD domain (VBP1-ΔC-ter) or the WD repeats 4–7 (VBP1-ΔW_{4-7}). Although the first WD motif seems to be particularly important for interaction with Vpu, all seven WD repeats as well as the C-terminal end are required for optimal binding. The N-terminal domain of h-βTrCP comprising the F-box motif (h-βTrCP-Δ7W) did not interact with Vpu.

Serine Residues 52 and 56 of Vpu Are Required for Both CD4 Degradation and Interaction with h-βTrCP

Serine residues 52 and 56, located in the hinge region between the two helices of the cytoplasmic domain of Vpu, are phosphorylated by casein kinase II (CKII) and are required to promote CD4 degradation (Schubert and Strebel, 1994; Paul and Jabbar, 1997). These residues are also required for binding of Vpu_c to h-βTrCP, since the double mutant Vpu_c S52N-S56N ($Vpu_{c\text{-}2/6}$) has lost its ability to interact with h-βTrCP (Figure 1A, lane 3). Vpu and h-βTrCP were transiently expressed in HeLa cells, and complexes were identified by coimmunoprecipitation using an anti-βTrCP polyclonal antiserum (Figure

Table 1. Analysis of the Ability of h-βTrCP Deletion Mutants to Interact with Vpu_c in the Two-Hybrid System

h-βTrCP deletion mutants	Interaction with Vpu_c
h-βTrCP: F 1 2 3 4 5 6 7	+++
VBP1: 1 2 3 4 5 6 7	+++
VBP1-ΔW1: 2 3 4 5 6 7	-
VBP1-ΔC-ter: 1 2 3 4 5 6 7	+
VBP1-ΔW4-7: 1 2 3	+
h-βTrCP-Δ7W: F	-

The h-βTrCP deletion mutants indicated and Vpu_c were fused to Gal4AD and Gal4BD, respectively. Interactions were assayed by growth in the absence of histidine and β-galactosidase production. Interaction strength measured as β-galactosidase produced was scored as strong (+++), weak (+), or negative (−).

3A, left panel). Consistent with the results of the two-hybrid screen, wild-type Vpu (lane 1) but not $Vpu_{2/6}$ (lane 3) was able to coprecipitate with h-βTrCP. Truncation of the F-box domain by deletion of amino acid residues 32–179 (h-βTrCPΔF) did not affect interaction with Vpu as evidenced by coimmunoprecipitation of h-βTrCPΔF with wild-type Vpu (lane 4) but not with $Vpu_{2/6}$ (lane 5). Vpu or $Vpu_{2/6}$ were immunoprecipitated with an AIDS patient serum, showing comparable levels of expression (Figure 3A, right panel, lanes 6–10). These results confirm that the interaction between h-βTrCP and Vpu_c detected in the two-hybrid system reflects the association of the two native proteins in cells.

To assess whether Vpu-βTrCP interaction can take place in vitro, the two proteins were cotranslated in rabbit reticulocyte lysate (RRL). Vpu–βTrCP complexes formed in vitro were identified by coimmunoprecipitation using the anti-peptide 553–569 anti-h-βTrCP Abs (Figure 3B, lane 2). In the absence of h-βTrCP, Vpu was not recognized by the anti-h-βTrCP antiserum (lane 1), but was precipitated by an anti-Vpu antiserum (lane 5). Mutation of the two phosphoserine residues in $Vpu_{2/6}$ again abolished Vpu-h-βTrCP interaction (lane 4).

Thus, Vpu and βTrCP were found to interact in three independent assays. In all of these assays, Vpu–h-βTrCP interaction required the presence of the two conserved phosphoserine residues in Vpu_c. Because of the crucial importance of these residues for the ability of Vpu to induce CD4 degradation, it is conceivable that binding of Vpu to h-βTrCP represents an obligate step for targeting CD4 into a degradative pathway.

Vpu Recruits h-βTrCP to Membranes

The association of Vpu with CMM is essential for its ability to induce CD4 degradation (Chen et al., 1993). The possible involvement of h-βTrCP in CD4 degradation would therefore require the association of βTrCP with membranes. Human βTrCP, translated in the absence of membranes, was incubated with CMM containing or not pretranslated Vpu. Reaction mixtures were then separated by centrifugation into membrane (m) and supernatant fractions (s) and analyzed. As expected, wild-type Vpu and $Vpu_{2/6}$ quantitatively partitioned with the membrane fraction (Figure 4A, lanes m; 4, 6, 10, and 12). Human βTrCP had no inherent affinity to membranes in the absence of Vpu (lane 2) and was found almost quantitatively in the membrane-free supernatant (lane 1). In the presence of wild-type Vpu, the majority of h-βTrCP associated with the membrane fraction (compare lane 4 to lane 3). The $Vpu_{2/6}$ phosphorylation mutant was unable to recruit h-βTrCP to the membrane fraction (lanes 5 and 6). This suggests that recruitment of βTrCP to membranes results from specific posttranslational association with membrane-bound phosphorylated Vpu. Consistent with the results from Figure 3A, deletion of the F-box domain in h-βTrCP did not affect the association of h-βTrCPΔF with membrane-bound Vpu (lanes 7–12).

Vpu Forms Ternary Complexes with h-βTrCP and CD4

The ability of Vpu to independently interact with CD4 or h-βTrCP requires that both proteins can simultaneously interact with Vpu and form stable complexes. To directly demonstrate CD4–Vpu–h-βTrCP ternary complexes, HeLa cells were cotransfected to express CD4 in combination with wild-type or mutant forms of both Vpu and h-βTrCP. After metabolic labeling, cell lysates were immunoprecipitated using a polyclonal antiserum directed against the ectodomain of CD4. As reported earlier (Bour et al., 1995b), wild-type Vpu and $Vpu_{2/6}$ were able to form complexes with CD4 identified by coimmunoprecipitation (Figure 4B, lanes 1 and 3). When full-length h-βTrCP or the F-box deletion mutant h-βTrCPΔF were expressed with Vpu and CD4, both βTrCP and h-βTrCPΔF were coimmunoprecipitated in the presence of wild-type Vpu (lanes 4 and 6) but not when $Vpu_{2/6}$ was present instead (lanes 5 and 7). As expected, h-βTrCP or h-βTrCPΔF did not coimmunoprecipitate with CD4 in the absence of Vpu (data not shown). Therefore, the presence of ternary complexes consisting of Vpu, CD4, and βTrCP clearly demonstrates that Vpu acts as a linker molecule between CD4 and βTrCP. The inability of $Vpu_{2/6}$ to act as such a linker again highlights the functional significance of Vpu phosphorylation.

Human βTrCP Interacts with Skp1p

Recent studies have shown that the F box is a motif found in multiple proteins such as cyclin F, Cdc4p,

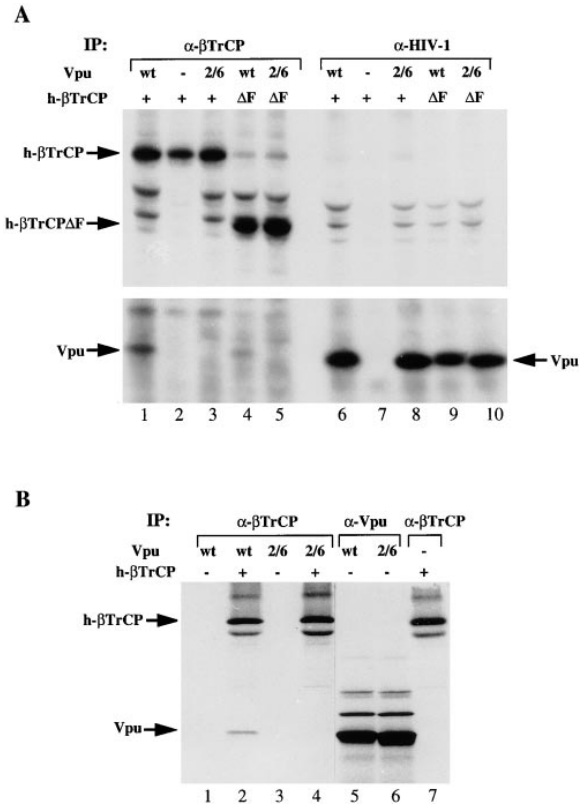

Figure 3. Human βTrCP and Vpu Interact Both In Vivo in Hela Cells and In Vitro

(A) h-βTrCP interacts with Vpu in HeLa cells. HeLa cells were co-transfected with plasmids expressing h-βTrCP (pcDNATrCP) and either wild-type Vpu (pNL-A1; lanes 1 and 6), no Vpu (pNL-A1/Udel; lanes 2 and 7), or the Vpu phosphorylation mutant $Vpu_{2/6}$ (pNL-A1/U2/6; lanes 3 and 8). HeLa cells were similarly transfected with plasmids expressing h-βTrCPΔF (pcDNATrCPΔF) plus either wild-type Vpu (lanes 4 and 9) or $Vpu_{2/6}$ (lanes 5 and 10). Cells were labeled with ^{35}S-translabel, lysed, and immunoprecipitated with the anti-h-βTrCP specific polyclonal antiserum (lanes 1–5) or with an HIV-positive patient serum, recognizing Vpu (lanes 6–10). The positions of βTrCP, βTrCPΔF, and Vpu are shown.

(B) Vpu and h-βTrCP interact in vitro. Vpu (lanes 1, 2, and 5) or $Vpu_{2/6}$ (lanes 3, 4, and 6), and h-βTrCP (lanes 2, 4, and 7) were in vitro translated either alone or cotranslated (lanes 2 and 4) as indicated, then immunoprecipitated with anti-Vpu (lanes 5 and 6), or anti-h-βTrCP anti-peptide 553–569 Abs (lanes 1–4 and 7), and analyzed in 15% SDS–PAGE.

Skp2p, or Grr1p. The function of these factors presumably is to promote degradation of cell cycle regulators by providing a link to the ubiquitin proteolysis machinery through F-box-dependant physical interaction with Skp1p (Bai et al., 1996; Li and Johnston, 1997). Thus, we investigated the potential association of h-βTrCP with human Skp1p (gift from S. Elledge) by two-hybrid assay. As shown in Figure 5, h-βTrCP interacts with Skp1p (lane 1), and as expected, the binding domain responsible for this interaction is located at the N terminus of h-βTrCP (h-βTrCP-Δ7W, lane 2). Deletion mutants of h-βTrCP lacking an intact F-box motif such as VBP1 or h-βTrCP-ΔF, are unable to interact with Skp1p (lane 3 and data not shown). Surprisingly, by quantitative assay based on β-galactosidase production, we found that interaction of Skp1p was almost seven times weaker with full-length h-βTrCP than that detected with the F-box N-terminal region alone (right panel, compare lanes 1 and 2). We also performed a two-hybrid screen using the F-box region h-βTrCP-Δ7W as a bait and found cDNA clones that corresponded to human Skp1p. The interaction between h-βTrCP and Skp1p was confirmed in vitro using GST-Skp1p fusion protein and in vitro translated h-βTrCP (data not shown). Altogether, these results demonstrate that h-βTrCP is a ligand of Skp1p through its F-box motif. However, the binding site for Skp1p is somehow partially masked in the context of full-length h-βTrCP and may require a conformational change for optimal binding. CD4c by itself did not interact with Skp1p (Figure 5, lane 4), suggesting again that Vpu is needed to link CD4 to h-βTrCP–Skp1p complexes.

Human βTrCP Deleted in the F Box Inhibits Vpu-Mediated CD4 Degradation

To demonstrate the role of βTrCP in the proteolysis of CD4, we attempted to interfere with its function by expressing the F-box deletion mutant h-βTrCPΔF, which was unable to bind to Skp1p but retained normal interaction with Vpu, through the seven WD repeats. If h-βTrCP is required for Vpu-mediated CD4 degradation, one would expect a dominant inhibitory effect of h-βTrCPΔF on CD4 degradation, as a result of competition with endogenous h-βTrCP for recruitment of Vpu.

We employed the in vitro CD4 degradation assay (Chen et al., 1993) supplemented with either full-length h-βTrCP or the h-βTrCPΔF mutant. CD4 was pretranslated in the presence of CMM followed by the synthesis of Vpu as reported earlier (Chen et al., 1993). The translation reactions were stopped, and lysates containing full-length h-βTrCP or h-βTrCPΔF translated in separate reactions were added to the Vpu-CD4 reaction mixtures (Figure 6A, panels a and b, respectively). As a negative control, mock-translated RRL was added to one reaction (Figure 6A, panel c). The amounts of CD4 present at each time point was analyzed and quantified using a phosphor Imager (Figure 6B). The rate and the kinetics of CD4 degradation, in the presence or in the absence of full-length h-βTrCP, were comparable (see panels a and c), with about 50% of CD4 being degraded within 40–90 min of incubation (Figure 6B). In contrast, CD4 degradation was significantly inhibited in the presence of h-βTrCPΔF (compare panel b with panels c and a), and more than 80% of CD4 was recovered after 90 minutes of incubation (Figure 6B). As previously reported, $Vpu_{2/6}$, which is inactive for CD4 degradation (Chen et al., 1993; Bour et al., 1995b; Margottin et al., 1996) and which does not interact with h-βTrCP (Figure 3), did not promote CD4 degradation in this assay, even in the presence of intact h-βTrCP (data not shown). Thus, expression of h-βTrCP by itself did not have a direct destabilizing effect on CD4. The lack of enhancement of Vpu-mediated CD4 degradation by h-βTrCP (compare panels a–c) suggests that the endogenous rabbit βTrCP detected by anti h-βTrCP Abs in RRL (see Figure 1C) is fully functional for CD4 degradation. From

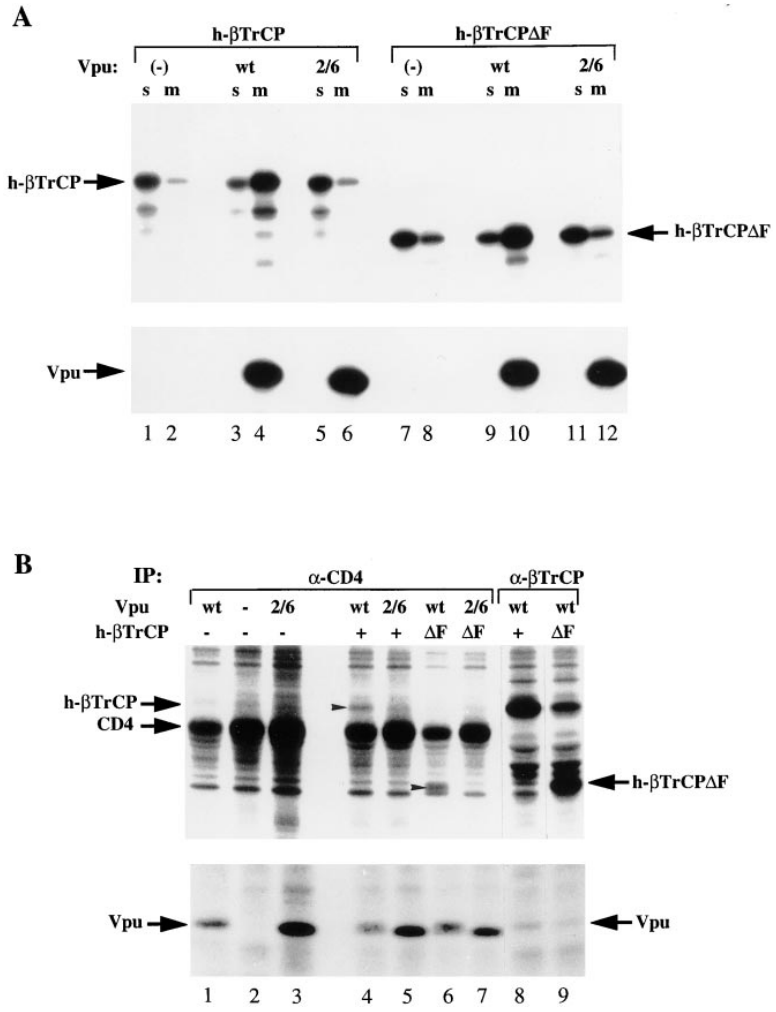

Figure 4. Recruitment of h-βTrCP to Membranes and Detection of CD4–Vpu–h-βTrCP Ternary Complex

(A) Vpu but not Serine phosphorylation mutant $Vpu_{2/6}$ is capable of recruiting h-βTrCP or h-βTrCPΔF in microsomal membranes. Vpu or $Vpu_{2/6}$ in the presence of canine microsomal membranes (CMM) and either h-βTrCP (lanes 3–6) or h-βTrCPΔF (lanes 9–12) were first in vitro translated separately and then mixed and incubated. Membrane-associated proteins from pellets (m) and soluble proteins from supernatants (s) were analyzed with 12% SDS–PAGE. In the absence of Vpu, h-βTrCP (lanes 1 and 2) or h-βTrCPΔF (lanes 7 and 8) are found essentially in the soluble fractions.

(B) h-βTrCP forms ternary complexes with Vpu and CD4. HeLa cells were cotransfected with plasmids encoding CD4 and either wild-type Vpu (lane 1), no Vpu (lane 2), or $Vpu_{2/6}$ (lane 3). For the analysis of ternary complexes between h-βTrCP, Vpu, and CD4, HeLa cells were triple-transfected to express CD4 and wild-type Vpu plus h-βTrCP (lanes 4 and 8) or h-βTrCP-ΔF (lanes 6 and 9). As controls, CD4 was coexpressed with the phosphorylation mutant $Vpu_{2/6}$ and either wild-type h-βTrCP (lane 5) or h-βTrCPΔF (lane 7). Cells were labeled with ^{35}S-translabel, lysed, and immunoprecipitated with a CD4-specific antiserum (lanes 1–7) or with the anti-h-βTrCP polyclonal antiserum (lanes 8–9). The positions of full-length h-βTrCP (lane 4) and h-βTrCPΔF (lane 6) are marked by arrows as identified by immunoprecipitation with the h-βTrCP-specific polyclonal antiserum (lanes 8 and 9).

these results, we can conclude that h-βTrCP or its functional homolog present in rabbit reticulocyte lysate is involved in connecting CD4 to a proteolytic pathway using Vpu as an adapter molecule. We further confirm that sequences in the N-terminal part of βTrCP containing the F-box motif are required to trigger downstream events leading to CD4 degradation.

Discussion

In this study, we have characterized a novel human protein that specifically interacts with HIV-1 Vpu both in vitro and in vivo. The protein identified as human βTrCP based on its homology to *X. laevis* βTrCP is a member of the WD-repeat proteins family. Human βTrCP mutants carrying deletions in the WD domain were unable to bind to Vpu, thereby identifying this domain as the binding site for Vpu. In addition, h-βTrCP contains an F-box motif at its N terminus that is a binding site for Skp1p. In yeast, Skp1p was found to play a role in the ubiquitin-dependent proteolysis of cell cycle regulator proteins such as the Cdk inhibitor Sic1p. Skp1p functions by providing a link between these proteins and the cellular ubiquitin-dependent proteolysis machinery. Our finding is thus consistent with a role of h-βTrCP as a link between Vpu–CD4 complexes and the ubiquitin-dependent cellular proteolysis machinery. Additional support for the involvement of h-βTrCP–Skp1p interaction in ER degradation of CD4 comes from our observation that deletion of the F-box N-terminal domain of

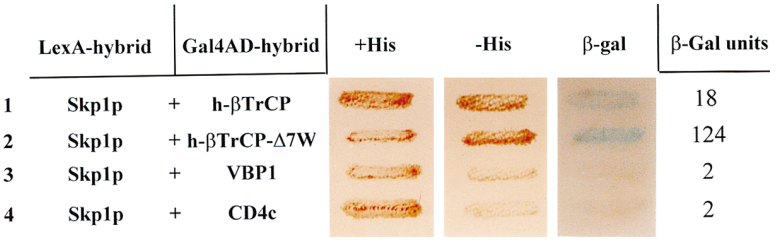

Figure 5. Specific Interaction of h-βTrCP with Human Skp1p in the Two-Hybrid System

Interactions with Skp1p of full-length h-βTrCP (lane 1), h-βTrCP WD deletion mutant (lane 2), the h-βTrCP WD-repeats region alone VBP1 (lane 3), or the cytoplasmic domain of CD4 (lane 4) were analyzed for histidine auxotrophy and qualitative and quantitative β-galactosidase expression in the L40 yeast strain.

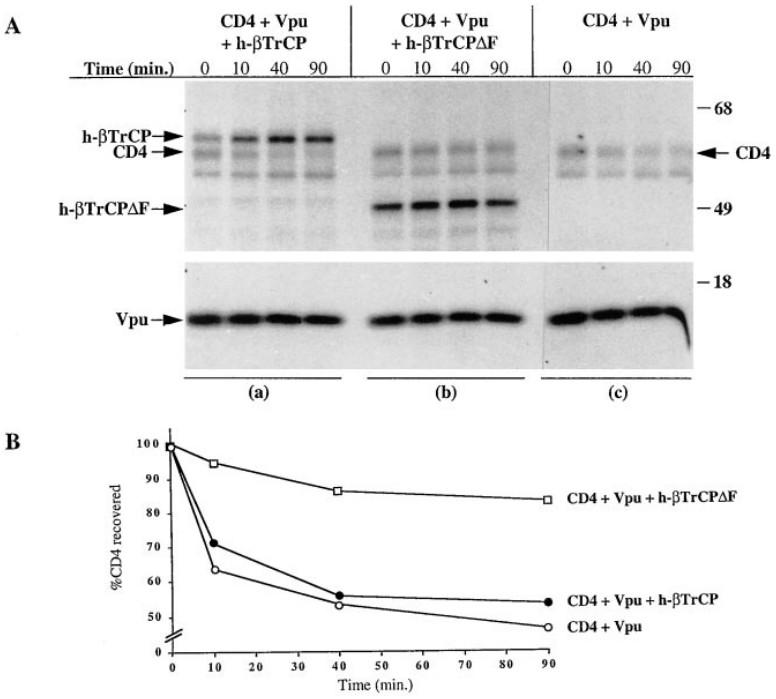

Figure 6. Transdominant-Negative Effect of h-βTrCPΔF on Vpu-Induced In Vitro CD4 Degradation

(A) After in vitro translation of CD4 in the presence of CMM, reaction mixture was supplemented with fresh reticulocyte lysate, incubated with Vpu RNA transcripts. Then, translation was stopped with RNase A and samples were supplemented with separately translated h-βTrCP (a), or h-βTrCPΔF (b) or reticulocyte lysate alone (c), and incubated at 30°C for CD4 degradation to occur. Aliquots were removed at the indicated times, and membrane-associated proteins were analyzed in 12% SDS–PAGE and autoradiography.

(B) Quantification of CD4 degradation. Scanning of the gel shown in (A) was performed using a phosphor Imager. The ratio of the amounts of CD4 left undegraded at each time point relative to the starting material was plotted as a function of time.

h-βTrCP resulted in a protein capable of interfering negatively with CD4 proteolysis in vitro. This suggests that the ability of h-βTrCP–Vpu–CD4 complexes to interact with Skp1p is necessary for targeting CD4 into a proteolytic pathway.

The two conserved phosphoserine residues (Ser-52/Ser-56) located in the cytoplasmic domain of Vpu are known to be essential for the ability of Vpu to induce CD4 degradation, yet they are not required for Vpu interaction with CD4. Our data now demonstrate that the role of the two phosphoserine residues in Vpu is to provide a binding site for h-βTrCP. This suggests that binding of Vpu–CD4 complexes to h-βTrCP is an obligate step in ER degradation of CD4. The demonstration of ternary complexes of CD4, Vpu, and h-βTrCP indicates that the binding sites in Vpu for CD4 and h-βTrCP are distinct and nonexclusive and provides direct evidence for the function of Vpu as an adapter molecule that links CD4 to h-βTrCP.

Human βTrCP has a modular organization with an F-box motif at the N terminus involved in interaction with Skp1p and WD repeats at the C terminus presumably needed for recruitment of substrates. Human βTrCP shares this general organization with at least eight members of the F-box-containing protein family, including the cell cycle regulator Cdc4p and the transcriptional regulator Met30p. In *S. pombe*, a new member of this WD and F-box family has been recently described, Pop1, which also mediates degradation of a CDK inhibitor, Rum1, through the ubiquitin proteolysis pathway (Kominami and Toda, 1997). Other members of the F-box protein family, such as Skp2p or Grr1p, contain leucine-rich instead of WD repeats (Bai et al., 1996; Li and Johnston, 1997).

We found that Skp1p interacted more efficiently with a C-terminally truncated form of h-βTrCP, containing the F-box motif but lacking the WD repeats, than with the full-length protein. It is thus conceivable that optimal interaction of Skp1p with normal h-βTrCP may require unmasking of a binding site in h-βTrCP. This could be triggered by the interaction of h-βTrCP with ligands of the WD repeats such as Vpu, resulting in a conformational change. The obvious benefits of such a "by-demand-only" mechanism for h-βTrCP interaction with Skp1p would be to make Skp1p available for targeting by other F-box-containing proteins and to prevent a constitutive substrate ubiquitination. In contrast, it was reported recently that both the F-box motif and the leucine-rich repeats were required for Grr1 interaction with Skp1p (Li and Johnston, 1997).

Based on previous work as well as the data presented in this study, we propose the following model for ER degradation of CD4 (Figure 7): HIV infection of $CD4^+$ cells and subsequent expression of the Env glycoprotein precursor gp160 result in the formation of stable CD4-Env complexes that are trapped in the ER. Vpu expressed in the ER interacts with a membrane-proximal domain of the cytoplasmic tail of CD4 (a) and recruits h-βTrCP to the membrane (b). The CD4–Vpu–h-βTrCP ternary complex recruits Skp1p (c), resulting in substrate ubiquitination (d) with subsequent degradation of CD4. The Env precursor gp160 has no role in this process other than delaying ER export of CD4, thereby increasing the efficiency of Vpu action. Because of the requirement for Vpu, ER degradation of CD4 is limited to HIV-1-infected cells. In normal cells, CD4 does not interact with h-βTrCP or Skp1p and is therefore not targeted into an ER degradation pathway. Our results suggest that Skp1p, besides its role in targeting regulators of the cell cycle to degradation, may be involved in various pathways of the proteolysis machinery, including ER-associated degradation (ERAD). Interestingly, it has recently been demonstrated that Skp1p is also involved in glucose-induced gene expression in yeast through interaction with Grr1 (Li and Johnston, 1997).

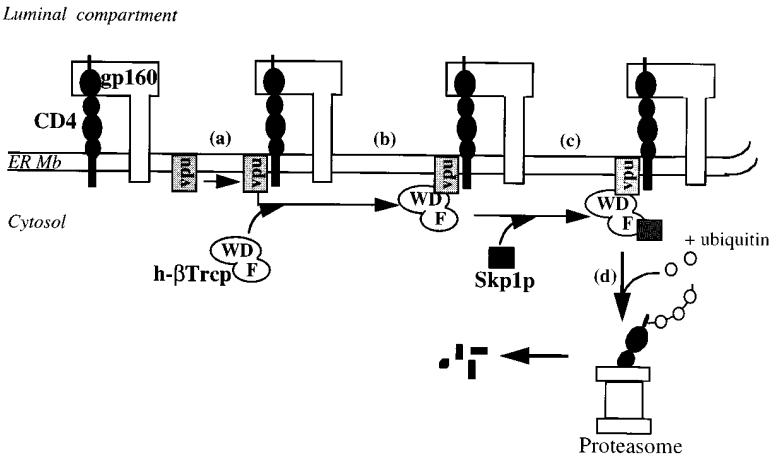

Figure 7. Model for Vpu-Mediated CD4 Targeting to the Proteasome

In infected cells, CD4 is retained in the endoplasmic reticulum through a complex with the envelope protein gp160. Vpu, complexed to CD4 (a), recruits h-βTrCP to the membrane through interaction with h-βTrCP WD repeats (b) and h-βTrCP N-terminal domain, containing the F box, interacts with Skp1p (c). This network of interactions, allowing h-βTrCP and Skp1p to be connected indirectly with CD4 through Vpu, leads to CD4 degradation via the proteasome (d).

It has been well established that normal cells have a quality control mechanism that enables them to eliminate aberrant or misfolded proteins from the ER in an ERAD pathway (Klausner and Sitia, 1990). This involves, at least in some cases, ubiquitination of the target proteins and the activity of proteasomes (reviewed by Brodsky and McCracken, 1997). In particular, the sensitivity to inhibitors of proteasomes suggests an involvement of cytosolic components in ER degradation and raises the question of how ER-associated proteins are degraded by cytosolic proteasomes. While dislocation of ER proteins to the cytosol is clearly a mechanism used for proteasomal targeting, some proteins, such as CFTR (Ward et al., 1995), may be targeted directly by ER membrane-associated proteasome complexes that were identified by immunoelectron microscopy (Rivett, 1993).

Whether ER degradation of CD4 involves dislocation of the protein into the cytoplasm, direct targeting of the cytosolic component of CD4 by proteasomes, or a different, yet undefined mechanism remains unclear. However, targeting of CD4 into a ubiquitin-proteasome pathway through an association of Vpu–CD4 complexes with Skp1p via h-βTrCP is consistent with the recent observation that Vpu-mediated degradation of CD4 involves the ubiquitin pathway and is sensitive to inhibitors of cytosolic proteasomes (Fujita et al., 1997; Schubert et al., 1998). In the presence of proteasome inhibitors, CD4 remains in the secretory pathway and is normally transported to post-ER compartments. It has to be determined whether Vpu could also function by catalyzing a translocation of CD4 to the cytosol similar to that performed by the HCMV *US2* and *US11* gene products. There are also interesting parallels between the Vpu-induced degradation of CD4 and the degradation of p53 by the human papillomavirus types 16 and 18 E6 protein (Scheffner et al., 1990). In both cases, degradation requires the formation of a ternary complex between the viral protein (Vpu or E6), a cellular factor (which in the case of Vpu is h-βTrCP and in the case of E6 is E6-AP), and the substrate (CD4 or p53). Neither Vpu nor E6 are affected by the subsequent degradation of the target molecules. While the E6 and E6-AP complex was found to function as a ubiquitin-protein ligase (Scheffner et al., 1993) that targets p53 and results in proteolysis of the ubiquitinated product, we have so far been unable to detect direct ubiquitination of CD4. It is possible, however, that such intermediates are too short-lived for detection. There is indirect evidence to suggest that CD4 degradation indeed involves the ubiquitin pathway. For example, mutation of potential ubiquitination sites in the cytoplasmic domain of CD4 or thermal inactivation of the E1 ubiquitin-conjugating enzyme were found to inhibit CD4 degradation (Schubert et al., 1998). It is therefore possible that, similar to the E6/E6-AP complex, Vpu/h-βTrCP complexes function as a ubiquitin-protein ligase that catalyze the ubiquitination of CD4. While this manuscript was submitted, papers by Feldman et al. (1997), Skowyra et al. (1997), and Verma et al. (1997) were published showing that Sic1p is targeted for degradation by a multisubunit complex including Cdc4, Skp1, and Cdc53. Cdc4 binds to the substrate Sic1p through its WD repeats only if this later is phosphorylated and interacts with Skp1 through its F-box motif. Thus, the behavior of βTrCP in regard to Vpu is analogous to that of Cdc4 toward Sic1p, suggesting that similar mechanisms are operating in degradation of cell cycle proteins and in Vpu-mediated degradation of ER-associated CD4.

Experimental Procedures

Two-Hybrid Screening, Cloning of h-βTrCP cDNA, Construction of Mutants, and h-βTrCP mRNA Expression

Two-hybrid screens were performed with a Jurkat cDNA library as described previously (Benichou et al., 1994), except that in the screen with the cytoplasmic domain of HIV-1_{LAI} Vpu (amino acid residues 28–81) fused to the Gal4BD in pGBT10, transformants were plated on medium without histidine and in the presence of 10 mM 3-amino-1, 2, 4-aminotriazole. β-galactosidase quantitative assays were performed as described (Bouhamdan et al., 1996). DNA sequencing was performed using an ABI 373A Automated Sequencer. H-βTrCP cDNA was amplified by nested PCR from 2 μg of library plasmid using two rounds of amplification, with appropriate primers. As a result of this procedure, a 1.4 kb fragment was subcloned in the pGAD-VBP1 plasmid as a 5′-Spe1-3′ BglII fragment, to reconstitute the pGAD-h-βTrCP clone. Alignments with homologous protein sequences were performed with the Macaw program (Schuler et al., 1991). VBP1 (corresponding to amino acid residues 251–569 of h-βTrCP) or the full-length h-βTrCP coding sequences were subcloned into pGBT9, pGEX4T2 (Pharmacia), or pCDNA3 (only for h-βTrCP) (Invitrogen) using standard procedures. VBP1-ΔW_1 (residues 292–569); VBP1-ΔW_{4-7} (residues 251–396) and VBP1-ΔC-ter (residues 251–545) mutants were constructed by PCR using appropriate primers. H-βTrCP-Δ7W (residues 1–291) was constructed by

inserting a SpeI–BglII fragment from h-βTrCP into pGAD1318, and βTrCPΔF was obtained by deletion of the AvrII–Asp-718 fragment of h-βTrCP (residues 32–179) with conservation of the reading frame. Human Skp1p (gift from S. Elledge) was subcloned in pLex10 for interaction analysis with h-βTrCP. Construction of the mutant $Vpu_{2/6}$ containing the double mutation S52N-S56N was described in Margottin et al. (1996). RNA expression was analyzed by Northern blot from multiple human cell lines (from Clontech), using a HindIII fragment from h-βTrCP as a 5′ probe or an EcoR1 fragment from VBP1 as a 3′ probe, according to the manufacturer's instructions.

Antibodies

Anti-peptide anti-h-βTrCP antibodies (Abs) purchased from Neosystem were raised in rabbits by immunization with two different synthetic peptides, 275–293 and 553–569. These Abs were affinity purified by adsorption on 30 μg of GST-VBP1 fusion protein, expressed in *E. coli* from pGEX-VBP1 and immobilized after electroblotting on nitrocellulose membrane. The purified Abs were eluted in glycine HCl (pH 3.0) and neutralized with 1 M Tris (pH 8.0). The polyclonal anti-h-βTrCP antiserum used in coimmunoprecipitation experiments was raised in rabbits after immunization with the N-terminal part of h-βTrCP (amino acids 3–192) expressed in *E. coli* using the pPLc24 vector as described (Strebel et al., 1986). An antiserum to the cytoplasmic domain of Vpu was produced as reported elsewhere (Maldarelli et al., 1993). The T4-4 CD4 and AIDS patient antisera were obtained from the AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH.

Metabolic Labeling and Immunoprecipitation

As previously described (Bour et al., 1995b), Hela cells cotransfected with the indicated plasmids expressing wild-type Vpu (pNL-A1), no Vpu (pNL-A1/Udel), or the Vpu phosphorylation mutant $Vpu_{2/6}$ (pNL-A1/U2/6), and either h-βTrCP (pcDNATrCP) or h-βTrCPΔF (pcDNATrCPΔF) were resuspended in methionine- and cysteine-free RPMI1640 and labeled for 30 min at 37°C with 200 μCi of Trans^{35}S-Label (10 mCi/ml; ICN Biochemical, Inc., Costa Mesa, CA). Cells were lysed in digitonin lysis buffer for 30 min at 4°C, precleared with 25 μl of protein A–Sepharose beads, and incubated with 3 μl of anti-βTrCP polyclonal antiserum. Immunoprecipitation was performed for 1 hr using 25 μl of protein A–Sepharose beads. Beads were rinsed three times in [0.1% digitonin, 150 mM KCl, 50 mM Tris-HCl (pH 7.4)], resuspended, boiled in 1% SDS sample buffer, and analyzed in 12% SDS–PAGE.

In Vitro Binding Studies

$HIV\text{-}1_{Lai}$ Vpu, $Vpu_{2/6}$, and h-βTrCP or h-βTrCPΔF deletion mutant subcloned in pCDNA3 under the control of the T7 promoter were translated in vitro for 90 min at 30°, either separately or cotranslated as indicated, in rabbit reticulocyte lysate, using ^{35}S-methionine and the coupled transcription/translation kit from Promega. Samples were then incubated for 60 min at 4°C in TENGN buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) with protein A–Sepharose and 3 μl of preimmune serum for preclearing, and immunoprecipitated overnight at 4°C either with anti-Vpu Abs, or with the anti-peptide 553–569 anti-h-βTrCP Abs. Immunoprecipitates were washed once in TENGN buffer (0.5% Nonidet P-40), washed twice in TENGN buffer (0.1% Nonidet P-40), and analyzed by SDS–PAGE and autoradiography.

Recruitment of h-βTrCP by Vpu to Membranes

Run-off transcripts of $HIV\text{-}1_{Lai}$ Vpu, $Vpu_{2/6}$, h-βTrCP and h-βTrCPΔF were synthesized according to Chen et al. (1993) prior to in vitro translation. In vitro translation of Vpu or Vpu2/6, in the presence of 2 μl of Canine microsomal membrane (CMM) (from Promega), and of h-βTrCP or h-βTrCPΔF without CMM, were first performed separately during 30 min at 30°C as indicated above. Then, in vitro translated Vpu or reticulocyte lysate alone used as a control and h-βTrCP were mixed, incubated 20 min at 30°C, diluted 3-fold in PBS, and centrifuged at 15,000 rpm for 10 min. Membrane-associated proteins in the pellet and soluble proteins in the supernatant were analyzed by 12% SDS–PAGE.

Degradation Assays of In Vitro Translated CD4

Degradation assays were performed as described previously (Chen et al., 1993), except that h-βTrCP or h-βTrCPΔF were translated in separate reactions and added to the CD4/Vpu translation mixture after CD4 and Vpu synthesis were stopped. CD4 was pretranslated in 25 μl for 45 min at 30°C in reticulocyte lysate containing CMM (Promega). Vpu RNA was then added with fresh reticulocyte lysate, and incubation was continued for 15 min at 30°C. Translation reactions were stopped by addition of RNase A to a final concentration of 1 mg/ml. Human βTrCP or h-βTrCPΔF pretranslated in 25 μl of lysate for 45 min at 30°C without CMM was then added, and CD4 degradation was analyzed on 12% SDS–PAGE and quantified on a Fuji FLA2000 Phorphor Imager at the indicated time points.

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EMBL Accession Number

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