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Misdirection of membrane trafficking by HIV-1 Vpu and Nef

Keys to viral virulence and persistence

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The HIV-1 accessory protein Nef is well known for its manipulation of host cell endosomal trafficking. By linking transmembrane proteins to endosomal coats, Nef removes them from the surface of infected cells. Modulation of MHC proteins leads to viral evasion of cellular adaptive immunity, whereas modulation of receptors for the HIV envelope glycoprotein, including CD4, enhances viral infectivity. The other HIV-1 accessory proteins, Vif, Vpr and Vpu, share a mechanism of action distinct from Nef in that each interacts with a multi-subunit ubiquitin ligase complex to target cellular proteins for proteosomal degradation. However, newly uncovered functions and mechanistic aspects of Vpu likely involve endosomal trafficking: these include counteraction of the innate antiviral activity of the cellular transmembrane protein BST-2 (tetherin), as well as the removal of the lipid-antigen presenting protein CD1d and the natural killer cell ligand NTB-A from the cell surface. This review focuses on how Nef and Vpu interfere with normal intracellular membrane trafficking to facilitate the spread and virulence of HIV-1.

Introduction

The chronic, persistent viral replication that characterizes untreated HIV-1 infection reflects the interplay of host defenses and viral countermeasures. To infect a new host, persistently replicate in that host for years, and spread to new individuals, HIV-1 must evade both innate defenses, including the so-called antiviral restriction factors and adaptive defenses, including antigen-specific cell-mediated immunity. To date, three restriction factors active against HIV-1 have been identified: APOBEC3 family proteins, TRIM5α and BST-2/tetherin. HIV-1 has evolved mechanisms to evade these factors, either by mutation of the targeted viral protein or by the acquisition of genes encoding specific proteins that antagonize them. Old world monkey TRIM5α causes premature uncoating of HIV-1 virion cores and consequently blocks the reverse transcription of the viral RNA genome in newly infected target cells. However, HIV-1 can replicate in human cells, because the viral capsid protein is not recognized efficiently by human TRIM5α.[1,2] The cytosine deaminases APOBEC3G and F cause hypermutation of viral DNA, but the HIV-1 accessory protein Vif links these proteins to the ubiquitin-proteasome system, thus degrading and antagonizing them.[3,4] The interferon-inducible protein BST-2/tetherin retains mature HIV-1 virions at the cell surface, restricting their release.[5,6] The viral accessory protein Vpu antagonizes BST-2 by removing it from its site of action on the cell surface; this enables the efficient release of progeny virions from the infected cell.[4,7-10] The HIV-1 accessory protein Nef is not yet known to antagonize a host cell restriction factor, although the Nef proteins of several SIVs, including SIVcpz, do counteract simian BST-2.[11-13] HIV-1 Nef nevertheless modulates several cellular transmembrane proteins to the advantage of the virus. Nef contributes to viral evasion of acquired cellular immunity by modulating major histocompatibility complex (MHC) proteins.[14-16] Nef also prevents cellular receptors for the viral envelope glycoprotein such as CD4 and CCR5 from interfering with viral assembly and release.[17-19]

The formation of a ternary complex between the viral accessory protein, the host restriction factor or other protein target, and a host co-factor (or several co-factors) is a common mechanism by which viral accessory genes neutralize host intrinsic and adaptive immunity. For example, counteraction of APOBEC3 proteins by Vif relies on the formation of a complex between Vif, APOBEC3, and a Cullin-5-containing ubiquitin ligase complex, with subsequent degradation of APOBEC3G by the proteosome. Similarly, the interaction between the viral accessory protein Vpr and the cullin 4A-DDB1 complex is presumably required for ubiquitination and degradation of an unidentified restriction factor. Like Vif and Vpr, Vpu and Nef can be considered as adaptors that link cellular targets to degradative pathways or to alternative trafficking pathways in the cell: Vpu by the induction of ubiquitination of its targets and Nef by linking its targets to endosomal coat proteins. However, unlike the other viral accessory genes, both Vpu and Nef are membrane-associated proteins. Moreover, unlike the known targets of the other viral accessory genes, the targets of both Vpu and Nef are transmembrane proteins. Here, we will focus on how Vpu and Nef manipulate intracellular trafficking of host cell transmembrane proteins to benefit viral spread and persistence.

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Functions

Nef. Nef is required for the rapid progression of primate lentiviral infections towards acquired immunodeficiency or AIDS.[20,21] Nef is expressed early during the viral replication cycle with the general purpose of converting the host cell into an efficient virus production and dissemination factory. Nef achieves this in part by reorganizing cellular signaling and trafficking machineries, while protecting the infected cell from immune surveillance long enough to ensure robust production of progeny virions. Although it has no enzymatic activity, Nef is involved in an apparent plethora of interactions with host cell proteins and acts as a molecular adaptor, establishing linkages between cellular proteins. The multiple functions of HIV-1 Nef have been extensively reviewed elsewhere.[22-26] Here, we will focus on functions that are related to the manipulation of intracellular trafficking. These functions involve modulation of the cell surface expression of specific receptors and the co-option of endosomal coat proteins and are listed in **Table 1**. In a few cases these receptors are upregulated to the cell surface, but in most cases they are downregulated from the cell surface by Nef.

Proteins upregulated by Nef include the membrane-anchored cytokines TNF and LIGHT, the dendritic cell-specific lectin DC-SIGN, and immature MHC-II bound to the invariant chain (MHC-II-Ii).[16,27,28] TNF and LIGHT are members of the TNF family that activate NF-κB and c-Jun N-terminal kinase pathways. The immediate advantage of upregulating TNF and LIGHT at the surface of the infected cell for the virus is not clear, but because TNF is a potent activator of HIV transcription, this might accelerate virus spread.[29] The lectin DC-SIGN binds HIV virions and promotes adhesion between dendritic cells and T cells. By increasing the cell surface expression of DC-SIGN, Nef facilitates the so-called trans-infection of T lymphocytes, in which viruses bound to dendritic cells are transferred to T cells.[28,30] Upregulation of MHC-II-Ii and downregulation of mature MHC-II lead to defective MHC-II-mediated peptide presentation and consequently impaired stimulation of $CD4^+$ T helper cells by HIV-infected cells.[16,31]

Downregulation of MHC-I (HLA-A and -B, but not -C and -E) protects HIV-infected cells from lysis by cytotoxic T lymphocytes and natural killer cells.[14,32] The CD1d molecule presents lipid antigens to CD1d-restricted natural killer T cells (NKTs). Whether any lipid antigens are specific to HIV-1 is unclear, but the downregulation of CD1d from the surface of dendritic cells could uncouple the interaction between these cells and NKTs and affect the subsequent initiation of cellular immune responses. The downregulation of the virus attachment and entry receptors CD4, CCR5, and CXCR4 protects the infected cell from superinfection, which would lead to premature cell death and inefficient virus replication.[19,33,34] These receptors can also interfere with the release of virus from the cell surface and impair the infectivity of released viruses due to their high binding affinity to Env.[18,35] In addition to CD4, another T cell co-receptor, CD8, is downregulated by Nef, presumably to avoid immune activation.[36] Macrophage mannose receptor can aid CD4-independent virus entry[37] and is required for efficient antigen internalization and MHC-II loading.[38] The removal of this receptor from the cell surface by Nef might impair superinfection, enhance virion release, and impair MHC-II-mediated antigen presentation.[39] Downregulation of the T cell receptor CD28 and its ligands in antigen-presenting cells, CD80 and CD86, may cause impaired activation of naïve T cells.[40,41] Finally, and perhaps not surprisingly given its many effects on trafficking, Nef alters the morphology of the endocytic compartment and the distribution of markers for membrane recycling compartments: Nef impairs recycling of transferrin receptor (TfR) and causes redistribution of the recycling center marker and regulatory GTPase Rab11.[42,43] Thus, trafficking defects caused by Nef lead to a broad range of benefits for the virus: from facilitated virus replication to immune evasion.

Vpu. Unlike Nef, HIV-1 Vpu is expressed late during the viral replication cycle, but like Nef, it plays an important role in viral virulence. The role of Vpu in vivo has been demonstrated using pigtailed macaques infected with SIV-HIV chimeric viruses (SHIVs) that express Vpu from HIV-1, since most SIVs lack a *vpu* gene. Infection of monkeys with SHIVs expressing no Vpu (or Vpu proteins with a scrambled transmembrane sequence or with inability to recruit a cellular β-TrCP-containing E3 ubiquitin ligase complex) yields virus levels in the blood that are at least 10-fold lower than infection with a SHIV encoding wild type Vpu; moreover, the animals infected with the *vpu* mutants have little or no loss of CD4-positive T lymphocytes, the hallmark of immunodeficiency in AIDS.[44-46] These effects are presumably due to the ability of Vpu to facilitate virion release and to contribute to immune evasion. On the molecular level, these activities have been linked to the downregulation of the cell surface levels of antigen-presenting complexes, T cell receptors and BST-2/tetherin as discussed below and summarized in **Table 1**.

Similarly to Nef, Vpu reduces CD4 levels at the cell surface, in this case not by affecting endosomal trafficking but by targeting newly synthesized CD4 for ubiquitination and an ERAD-related pathway at the ER;[47-51] this disables the delivery of CD4 to downstream compartments including the plasma membrane. The extraction of CD4 from the ER membrane by Vpu also ensures release of the viral envelope glycoprotein (Env) precursor from the ER, where it would otherwise be retained by CD4 due to the high binding affinity of these proteins for each other.[52] The targeting of CD4 to the proteasome depends on the interaction of Vpu with the SCF-E3 ubiquitin ligase complex subunit β-TrCP, which is mediated by a canonical DSGxxS motif in the cytoplasmic domain of Vpu.[53] Of the two isoforms of β-TrCP, $\beta\text{-TrCP}_2$ is the one required for Vpu-dependent degradation of CD4.[54] The interaction of Vpu with β-TrCP also causes the sequestration of this ubiquitin ligase complex from its natural substrates, resulting in the stabilization of certain cellular proteins. Such substrates include the regulators of transcription IκB and β-catenin.[55,56] While IκB inhibits the transcription factor NFκB and limits the expression of anti-apoptotic proteins, β-catenin is involved in cell adhesion and interactions with nuclear transcription factors that initiate the expression of various oncogenes. The immediate advantage of such effects to the virus is not clear, especially

Table 1. Effectors of Nef and Vpu that are modulated at the cell surface by altered intracellular membrane trafficking

	Target	Function of the target	Down/up-regulation	Motifs in Nef/Vpu required for modulation	Cellular co-factor complex corresponding to residues at left	Motifs in target required for modulation	Altered transport step	Selected references
Nef	1. TNF and LIGHT	Membrane-anchored cytokine	UP	$ExxxLL_{165}$	APs	N/D	Decreased internalization	27
	2. DC-SIGN	Dendritic cell-specific lectin, simultaneously binds virions and promotes adhesion with T cells	UP	$ExxxLL_{165}$	APs	LL_{15}	Decreased internalization	28, 149
	3. MHC-II-Ii (CD74)	Invariant chain-loaded (intermediate) MCH-II	UP	$ExxxLL_{165}$ DD_{175} EE_{155}	AP-2 V1H?/AP-2 ARF1/β-COP	L_7, L_{17}	Decreased internalization	16, 116, 150
	4. MHC-I (HLA-A and B, but not C and E)	Peptide antigen presentation	DOWN	$PxxPxxP_{78}$ $EEEE_{65}$ R17/R19 M20	SH3? PACS-1/2 or AP-1 β-COP N/D	Y_{320}SQAxxxD	Transport between TGN and late endosomes	14, 23, 87, 90, 119, 151-155
	5. MHC-II	Peptide antigen presentation	DOWN	$PxxPxxP_{78}$ $EEEE_{63}$ $ExxxLL_{165}$	SH3 PACS-1/2 or AP-1 APs	N/D	Sequestration in TGN?	16, 150
	6. Mannose receptor	Antigen capture on the surface of Macrophgaes, DCs	DOWN	N/D	N/D	LY_{1429} and LV_{1445} in the CD	Sequestration in TGN	39
	7. CD1d	Lipid antigen presentation	DOWN	$ExxxLL_{165}$ DD_{175} $PxxP_{75}$ $EEEE_{63}$	APs V1H/AP-2 SH3 PACS-1/2 or AP-1	Y_{331}QGV in the CD	Increased internalization	121
	8. CD4	Entry receptor	DOWN	$ExxxLL_{165}$ DD_{175} EE_{155} WL_{58}	AP-2 VIH/AP-2 ARF1/β-COP CD4 binding site	LL_{414}	Increased internalization, decreased recycling and rerouting to the MVB	17, 92, 126, 156-158
	9. CCR5	Entry co-receptor	DOWN	$PxxP_{75}$ $EEEE_{63}$	SH3 PACS-1/2 or AP-1	N/D	Increased internalization	19
	10. CD28	T cell activation by APC	DOWN	$ExxxLL_{165}$ WL_{58}	APs CD28 binding site?	LL_{186} in the CD (only SIV Nef)	Increased internalization	40
	11. CD80/86	Immune costimulatory receptor of APC	DOWN	120–125 aa region	PKC, Scr or RACK1?	CD	Redistribution of surface protein to the Golgi	41, 122, 123
	12. CD8	T cell receptor	DOWN	$ExxxLL_{165}$ DD_{175} WL_{58}	AP-1 V1H/AP-2 CD8 binding site?	FMK_{206} in the CD	Increased internalization at the PM	36, 120
	13. Transferrin receptor (CD71)	Transferrin uptake	DOWN	$ExxLL_{165}$	APs	N/D	Decreased recycling	43

Color code represents the type of effector by function: (1) pink, receptors that activate transcriptional regulation, (2) green, proteins with physical/mechanistic function, such as adhesion or virion-capture, (3) gray, antigen presenting molecules, (4) blue, T cell receptors involved in the immune response. Transferrin receptor is not known to be directly involved in immune functions. APC, antigen-presenting cells; MVB, multivesicular body; TGN, trans-Golgi network; PM, plasma membrane; CD, cytoplasmic domain; APs, adaptor protein complexes; Ub, ubiquitin; N/D, not determined.

since Nef upregulates NFκB while Vpu seems to do the opposite. These effects on protein stabilization by Vpu could be incidental byproducts of the interaction between Vpu and β-TrCP.

Vpu downregulates the interferon-inducible host restriction factor BST-2/tetherin from the cell surface by an incompletely described mechanism, which partially relies on Vpu-dependent

Table 1 (continued). Effectors of Nef and Vpu that are modulated at the cell surface by altered intracellular membrane trafficking

	Target	Function of the target	Down/up-regulation	Motifs in Nef/Vpu required for modulation	Cellular co-factor complex corresponding to residues at left	Motifs in target required for modulation	Altered transport step	Selected references
Vpu	1. CD40	VC-BL cell adhesion	UP	N/D	N/D	N/D	Increased protein level or transport to the cell surface	159
	2. BST-2/tetherin	Capture of mature virions at the cell surface	DOWN	$DSGxxS_{56}$ AxxAxxA face of TMD helix	β-TrCP BST-2 binding site	S and T residues in CD (Ub acceptors) Hydrophobic face in TMD	Decreased recycling, accumulation in TGN and recycling compartments	5, 6, 57, 61, 63, 104, 105, 135
	3. CD1d	Lipid antigen presentation	DOWN	TMD helix	CD1d binding site?	N/D	Exit from early endosomes during recycling	64
	4. CD4	Entry receptor	DOWN	DSGxxS L_{63}, V_{68}	β-TrCP N/D	$LSEKKT_{419}$ K, S and T residues in the CD (Ub acceptors)	ER-associated degradation	47, 50, 51, 53, 106, 160-62
	5. NTB-A	NK co-activating receptor on CD4+T cells	DOWN	TMD helix	N/D	TMD?	Endosomal sequestration?	65

Color code represents the type of effector by function: (1) pink, receptors that activate transcriptional regulation, (2) green, proteins with physical/mechanistic function, such as adhesion or virion-capture, (3) purple, antigen-presenting molecules, (4) blue, T cell receptors involved in the immune response. Transferrin receptor is not known to be directly involved in immune functions. APC, antigen-presenting cells; MVB, multivesicular body; TGN, trans-Golgi network; PM, plasma membrane; CD, cytoplasmic domain; APs, adaptor protein complexes; Ub, ubiquitin; N/D, not determined.

ubiquitination.[6,7,57-59] However, the degradation of BST-2 is not directly correlated with downregulation from the cell surface or with enhanced virion release in some experimental systems.[57,60-63] This raises the possibility that rather than simply degrading BST-2 via the ubiquitin-proteasome system, Vpu might modulate the intracellular trafficking of BST-2 as discussed below. Two recent studies identified novel targets of Vpu: CD1d and NTB-A, both of which are downregulated from the cell surface.[64,65] CD1d downregulation appears to be a function shared by Vpu and Nef.[64] Vpu alone downregulates the NTB-A receptor from the surface of HIV-1-infected T cells; this inhibits the lysis of infected cells by natural killer cells.[65] Neither the downregulation of CD1d nor of NTB-A involves degradation but seems instead to involve altered intracellular trafficking of these proteins.

Interestingly, whereas the effect of Nef on TfR suggests a generalized perturbation of traffic within endosomal pathways, Vpu does not affect TfR and thus alters endosomal traffic more specifically.[64,66] However, Vpu does delay secretion along the biosynthetic pathway, and this effect depends on the DSGxxS motif.[66] This mechanism might be involved in the Vpu-mediated downregulation of MHC-I from the cell surface,[67] as well as in the retention of BST-2 in the trans-Golgi network (TGN), as discussed below.

Other Vpu-interacting proteins include CAML, MHC-II-Ii, and UBP (all targets identified in yeast two-hybrid screens), and TASK-1 (an ion channel protein), whose transmembrane domain is partly homologous to that of Vpu. The functions of these interactions remain elusive. Although initially proposed as a cellular protein that Vpu antagonizes to enhance virion release, CAML appears to restrict virion release only in simian cells.[68,69] The interaction of Vpu with MHC-II-Ii may affect MHC-II-mediated antigen presentation.[70] Overexpression of UPB and the interaction of this protein with Gag results in limited virion release, thus the interaction of Vpu with UPB may disrupt the UBP-Gag association and facilitate virion release.[71] The interaction of Vpu with TASK-1 results in the disruption of the ion channel activities of both proteins and was proposed to mediate Vpu-dependent enhancement of virion release.[72,73] However, the ability of Vpu to enhance virion release is now ascribed to its interaction with BST-2/tetherin, an interaction that could in principle be inhibited by the overexpression of TASK-1.

Structure and Motifs Required for Trafficking Effects

Nef. HIV-1 Nef is typically 27 kDa in apparent mass and 206 amino acids in size. It is membrane-associated via N-terminal myristoylation and forms dimers. Myristoylation at Gly2 in at the N-terminal MGxxxS motif, as well as Asp123-dependent dimerization, are apparently essential for most Nef functions, although whether dimerization is the key functional attribute of Asp123 is not clear.[74,75] NMR spectroscopy and X-ray crystallography have revealed three structural parts of Nef: a flexible N-terminal region (residues 1–57), a C-terminal folded core (residues 58–149 and 181–206) and a flexible C-terminal loop (residues 150–180). A surface interacting with CD4 was determined by solution NMR and includes Trp57, Leu58, Glu59 and Arg106.[76] The same motif presumably interacts with CD8 and CD28, as mutating these residues, specifically Trp57 and Leu58, disrupts downregulation of these receptors.[36,40] The sites in Nef for interaction with other targets such as MHC-I and MHC-II are not known. However,

because Nef functions related to CD4 and MHC-I are genetically separable, the site for interaction with MHC-I is presumably elsewhere.

The motifs in Nef required for interaction with several cellular co-factors have also been characterized, and some of these are directly involved in endosomal trafficking (**Fig. 1**). An acidic motif (or cluster) $EEEE_{65}$ just N-terminal of the folded core binds to the phosphofurin acid cluster sorting protein 1 (PACS-1), which may mediate retrograde endosome-to-TGN transport.[77,78] Alternatively or in addition, an interaction with PACS-2 may trigger a multi-kinase cascade leading to the downregulation of MHC-I.[79] A contrasting model proposes that the $EEEE_{65}$ acidic cluster instead participates directly in the interaction of Nef with the μ subunit of the adaptor protein complex 1 (AP-1).[80,81] The polyproline domain including the $PxxP_{75}$ sequence of HIV-1 Nef binds the SH3 domains of Src kinases, including the monocyte-specific kinase Hck and the lymphocyte-specific kinase Lck.[82,83] The importance of the interactions between Src kinases and Nef for its trafficking functions is not clear, although signaling cascades involving an unidentified Src-family kinase have been proposed as important for the Nef-mediated modulation of MHC-I.[83] Moreover, a recent study suggests that Nef-induced activation and recruitment of Hck results in the perturbation of Golgi structure and Golgi-associated glycosylation, resulting in trafficking defects such as the reduced expression of the receptor for macrophage colony stimulating factor.[84] On the other hand, a nearby residue in the HIV-1 Nef polyproline helix, P78, is required for the downregulation of MHC-I, but it is not required for SH3-binding; this suggests that the polyproline domain of Nef might participate in the modulation of MHC-I by an alternative mechanism.[85] An acidic di-leucine motif $ExxxLL_{165}$ in the flexible C-terminal loop is required for the interaction with AP complexes and for the downregulation of CD4.[86,87] It is preceded by a diacidic sequence EE_{155}, which interacts with β-COP, possibly via the regulatory endosomal GTPase ARF1, and is required for optimal CD4 degradation.[88,89] An alternative interface for the interaction with β-COP is formed by residues arginine 17 and 19 and is required for the degradation of MHC-I.[90] Two aspartic acids succeeding the di-leucine motif, DD_{175}, are required for binding to V1H, the catalytic subunit of an endosomal ATPase involved in acidification.[91] However, this motif has also been shown to participate, along with the ExxxLL motif, in the direct binding of Nef to AP-2, the endosomal clathrin adaptor involved in endocytosis.[92]

Due to its apparent flexibility and large solvent-accessible surface area, Nef can potentially assume various conformations that expose different sets of interaction motifs and thus recruit functionally distinct co-factor complexes as illustrated in **Figure 2**.[26,93,94] Such alternative conformations could be adopted upon binding with specific target proteins.

Vpu. HIV-1 Vpu is typically 17 kDa in apparent mass and 81 amino acids in size. Like Nef, it is associated with membranes but via a single transmembrane domain (TMD) in a type I orientation. NMR studies have revealed a short N-terminal luminal domain (residues 1–3), a transmembrane α-helix (residues 4–27), two cytosolic α-helices (residues 32–49 and 57–72), a flexible connector loop (50-56), and a flexible C-terminal tail.[95-99]

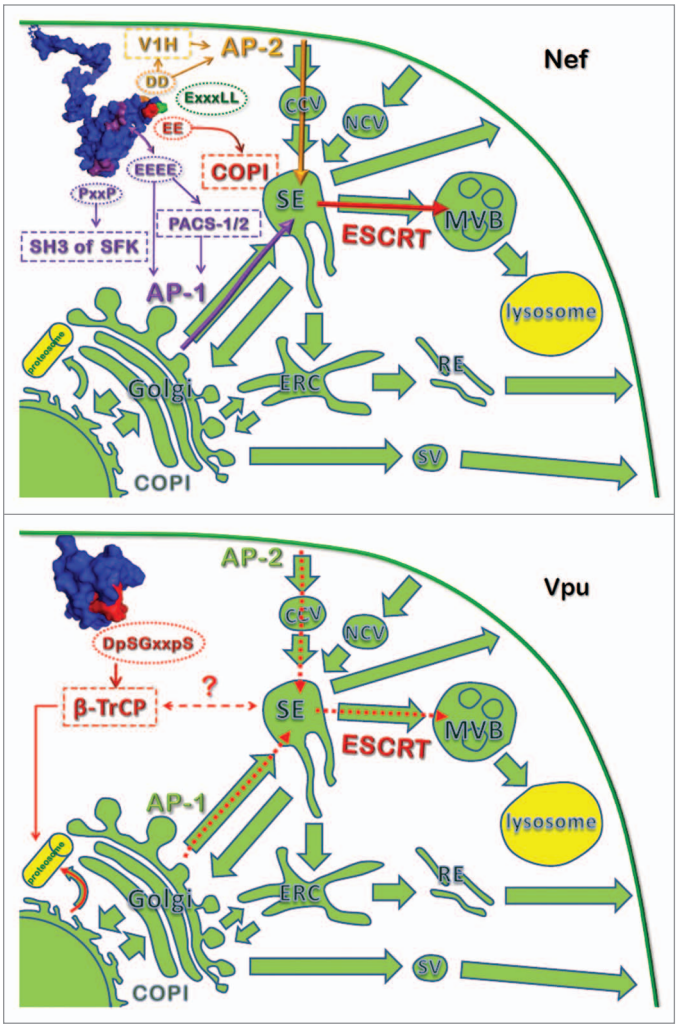

Figure 1. The effects of Nef and Vpu on intracellular trafficking. Top: Nef alters multiple intracellular trafficking steps. The ExxxLL motif in Nef directs interactions with AP complexes. Golgi-endosome-related trafficking and designated residues in Nef are depicted in purple; PM endosome in orange; endosome-MVB in red. SFK indicates unidentified Src-family kinase. Full length Nef molecule with the interaction motifs represents a composite of two solution NMR structures, Protein Data Bank ID's 2NEF and 1QA5,[146,147] assembled in PyMOL. Bottom: intracellular trafficking steps likely affected by Vpu. The cytosolic domain of Vpu with designated DpSGxxpS motif represents solution NMR structure 1VPU.[148] In both panels, wide green arrows represent normal intracellular trafficking pathways; other-colored narrow arrows represent trafficking stimulated by HIV-1 Nef or Vpu. Cellular structures: CCV, clathrin-coated vesicles; NCV, non-clathrin vesicles; SE, sorting endosome; MVB, multivesicular body; ERC, endosomal recycling center; RE, recycling endosomes; SV, secretory vesicles. Two structures involved in protein-degradation, proteosomes and lysosomes, are depicted in yellow.

The downregulation of NTB-A and BST-2 functionally maps to the TMDs of these proteins and the TMD of Vpu. An interaction between the TMDs of Vpu and BST-2 has been demonstrated by immunoprecipitation, FRET, and bi-molecular fluorescence complementation.[61,100-104] These data support a

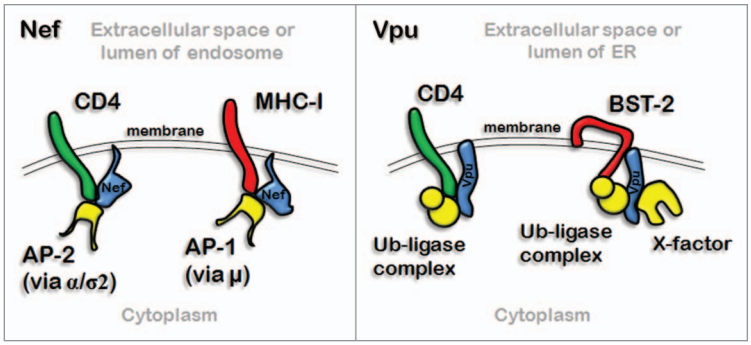

Figure 2. Tripartate complex formation between Nef or Vpu, cellular co-factors, and target proteins. Left: Nef binds differently to the AP-complexes depending on its target: when Nef downregulates CD4 it binds AP-2 via its ExxxLL motif and the α/σ2 subunits of AP-2; when Nef downregulates MHC-I it binds via unclear sequences to the μ subunit of AP-1, while directing a key tyrosine residue of MHC-I to the canonical binding site for Yxxϕ motifs on the μ subunit. Right: Vpu binds an E3 ubiquitin ligase complex, which results in ubiquitination and downregulation of CD4 and BST-2. "X" refers to unidentified factors that might bind the cytoplasmic domain of Vpu or BST-2; in the case of BST-2 such factors could recognize ubiquitin.

model in which the α-helices of the two proteins bind each other in an anti-parallel orientation. Unlike the case of Nef, only one Vpu-interacting cellular co-factor with meaningful function is currently known: β-TrCP. Phosphorylation of the two serines (Ser52 and Ser56) in the DSGxxS motif in the flexible region of the cytosolic domain of Vpu is required for the association of Vpu with the β-TrCP, which occurs via the WD domain of β-TrCP.[53] The interaction between Vpu and β-TrCP is required for the downregulation of CD4; it contributes to the downregulation of BST-2; and it is dispensable for the downregulation of NTB-A. These results prompt the continuing search for other Vpu-binding proteins, as well as for additional ways in which Vpu might interfere with the surface expression of cellular proteins independently of β-TrCP-mediated ubiquitination (indicated by "X-factor" in **Fig. 2**).

Additional sites with the potential to interact with cellular co-factors have been noted in the cytosolic domain of Vpu: these include a potential tyrosine-based AP-binding motif (Yxxϕ) in the membrane-proximal hinge region upstream of helix 1, which overlaps with a potential leucine-based motif (E/D)xxxLϕ, as well as residues L_{63} and V_{64} in helix 2, which could also comprise a leucine-based AP-binding motif. Whereas L_{63} is required for CD4 downregulation, the substitution R30A,K31A within the Yxxϕ sequence and the truncation of helix 2 affect Vpu's localization to the TGN and its ability to enhance virion release.[105,106] However, whether the aforementioned residues or regions are involved in the binding of Vpu to specific cellular co-factors is unclear.

Traffic Control

Major trafficking routes within the membrane systems of eukaryotic cells are depicted in **Figure 1** and represent potential sites of interference by viral proteins. In brief, cargo (i.e., transmembrane proteins) within the secretory pathway is synthesized in the endoplasmic reticulum (ER) and transported to the plasma membrane (PM) through successive compartments: first the Golgi, then the TGN and finally secretory vesicles. Once at the PM, certain receptors are subject to endocytosis, then transport through early/sorting endosomes and recycling endosomes. From both sorting and recycling endosomes, cargo can be sent directly back to the PM or be retrieved to the TGN. A distinct compartment, the endocytic recycling center, is identified by the presence of the regulatory GTPase Rab11 and is localized in the perinuclear region. Direct transport from sorting endosomes to the PM is referred to as fast recycling and takes 1–2 min; in contrast, recycling from the perinuclear recycling endosomes to the PM takes about 12 min.[107] From sorting endosomes, cargo can also be delivered to late endosomes or multivesicular bodies (MVB) and subsequently, to the lysosome.[108] Lysosomes, like proteosomes, are a major site of protein degradation. While lysosomes are a terminal branch of the endocytic pathway, proteosomal degradation of membrane proteins generally occurs after the protein is extracted from the ER and is thus linked to ER-associated degradation, a process that includes the degradation of misfolded proteins. Initial internalization of transmembrane proteins at the PM can be clathrin-dependent or -independent. These two pathways merge in the sorting endosome.[109] In the endosomal system, each cargo destination is defined by a specific set of factors that interact with the cargo either directly or indirectly: vesicle coat components such as clathrin AP complexes, vesicle fission factors, tethers, SNARE proteins, fusion factors, and small GTPases.[110] Specific AP complexes are responsible for the formation of clathrin coats and the recruitment of vesicle cargo at various post-Golgi membrane compartments. AP-2 is specifically associated with internalization at the PM, whereas AP-1 directs the transport between the TGN and endosomes and AP-3 directs cargo to late endosomes and lysosomes. The breadth and specificity of these pathways can be modulated by various factors, including clathrin-associated sorting proteins (CLASPs), some of which recognize ubiquitin as a sorting signal.[111] Ubiquitin can function as a endocytic signal, or it can bind various proteins of the endosomal sorting complex required for transport (ESCRT) to mediate cargo delivery to late endosomes and multivesicular bodies.[112-114] Nef and Vpu redirect their targets along these pathways within the endosomal system, essentially by co-opting several of these cellular regulatory mechanisms.

Nef. Nef localizes throughout the endosomal system including the PM but appears most concentrated in the juxtanuclear region of the cell.[115] To upregulate targets such as MHC-II-Ii or DC-SIGN, Nef likely sequesters AP complexes, particularly AP-2, away from the targets, such that the proteins are displaced to the PM by default.[116] To downregulate molecules from the cell surface, Nef alters their trafficking by recruiting endosomal coat proteins to the target. This activity requires the formation of ternary complexes between Nef, the target, and the endosomal coat proteins, specifically AP-1, AP-2, and β-COP.[26] The recruitment

of AP-2 stimulates the rate of endocytosis of CD4, whereas (at least in T cells), the recruitment of AP-1 results in the retention of MHC-I in the TGN[117] (**Fig. 2**). Recruitment of β-COP results in targeting to late endosomes and ultimately the partial degradation of CD4 and MHC-I in lysosomes.[88,90]

Several motifs in Nef are responsible for these interactions with endosomal coat proteins. The highly conserved ExxxLL motif in Nef is required for the binding of Nef to AP complexes,[86,87,118] whereas other motifs recruit additional factors such as β-COP and possibly ARF-1 (**Fig. 1**). Still others, specifically the $EEEE_{65}$ sequence, appear to allow Nef to bind AP complexes when in ternary complex with its target.[80] Two distinct models have been proposed by different research groups to explain the downregulation of MHC-I by Nef. One incorporates the interactions above; in this model Nef recruits AP-1 (and β-COP) to MHC-I, trapping MHC-I in the TGN and directing it to lysosomes for degradation.[80,90,117,119] Another model is more complex and invokes signaling interactions via unidentified Src-family kinase(s) as noted above, with ultimate modulation of protein sorting via PI-3-kinase.[83] Like CD4, the effects of Nef on CD8 and CD28 seem primarily to involve enhanced endocytosis and likely depend on AP-2 as a cellular cofactor, although recent RNA-interference experiments support a role for AP-1.[120] CD1d appears to be downregulated by both enhanced endocytosis and retention in the TGN.[121] CD80 and 86 appear to be redistributed to the TGN by Nef by an uncharacterized pathway that includes actin polymerization and is Rab11-dependent.[41,122,123] Notably, the modulation of CD1d, CD80, and CD86 by Nef has not been as extensively studied as has the modulation of CD4 and MHC-I, and recent data using chimeric molecules suggests that the cytoplasmic domains of CD1d, CD80, and CD86 are not well recognized by Nef.[120] SIV Nef proteins are now known to downregulate non-human primate BST-2; this occurs via the formation of a ternary complex between AP-2, Nef, and BST-2.[11-13,124] Interestingly, although Nef is not known to induce ubiquitination of its targets, it is itself ubiquitinated, and proteins of the ESCRT system are involved in the downregulation and targeting of CD4 by Nef to lysosomes.[125,126]

Overall, these data support the overarching notion that Nef co-opts endosomal coat proteins and trafficking pathways to misdirect cellular proteins away from the plasma membrane. An intriguing aspect of Nef's mechanism of action is that the ternary interactions formed between Nef, its target (MHC-I or CD4), and AP complexes (AP-1 in the case of MHC-I and AP-2 in the case of CD4) appear to be cooperative in nature.[80,127] For example, when Nef is fused to the cytoplasmic domain of MHC-I, binding of the fusion-protein to AP-1 (via the μ1 subunit) is more avid than the binding of either Nef or the cytoplasmic domain of MHC-I alone to AP-1.[80,81] Similarly, Nef binds via its ExxxLL motif to the α/σ2 hemicomplex (two of the four subunits of the AP-2 complex) more avidly in the presence of the cytoplasmic domain of CD4. Moreover, these cellular targets of Nef have sequences that are "would be" AP-binding motifs within their cytoplasmic domains, and these are required for modulation by Nef. For example, the cytoplasmic domain of MHC-I contains a tyrosine required for Nef-responsiveness but whose sequence context lacks the hydrophobic residue typical of Yxxϕ AP-binding motifs. Similarly, the cytoplasmic domain of CD4 contains a required dileucine whose sequence context lacks the acidic residue(s) typical of ExxxLϕ AP-binding motifs. Nef appears to facilitate the interaction of these "would be" binding sequences in its targets to the AP complexes; thus, Nef can be regarded conceptually as a virally encoded CLASP, which alters the breadth and specificity of clathrin-mediated endosomal trafficking to the advantage of the virus.[80]

Vpu. Despite its ability to downregulate target proteins from the cell surface, Vpu proteins of HIV-1 subtype B, which are commonly used in many laboratories, display predominantly intracellular rather than PM localization; that is, Vpu resides in the ER, TGN and endosomes.[105,128-130] Residence of Vpu in the ER is consistent with its ability to degrade CD4 by an ERAD-related mechanism. Vpu could potentially interfere with BST-2 trafficking at any of the above compartments, but localization to the TGN has been associated with Vpu-mediated enhancement of virion release.[105]

Ion channel formation. Vpu forms ion channels via oligomerization of its TMD.[131] This activity is separable from CD4 downregulation, which maps to the cytoplasmic domain (CD) of Vpu, but overlaps with anti-BST-2 activity, insofar as both require an intact TMD. Ion channel formation could in principle affect the endosomal transport of BST-2, but recent data indicate that certain amino acid substitutions associated with loss of channel activity do not impair the downregulation of BST-2 or the enhancement of virion release.[104,132] While this leaves little room for the role of ion channel formation during the downregulation of BST-2, channel activity could still play a role in the modulation of other targets such as CD1d.

Vpu serine residues 52 and 56 of the DSGxxS motif; β-TrCP; and the ubiquitination and degradation of BST-2. Much effort has been invested in assessing the role of protein degradation in the Vpu-dependent downregulation of BST-2, as well as the roles of Vpu serine residues 52 and 56 and β-TrCP in these processes. Nevertheless, controversy concerning these subjects persists.

Degradation. At first reported as the principle effect underlying Vpu-mediated downregulation of BST-2 and the counteraction of restricted virion release,[132] the degradation of BST-2 is instead emerging as a potential secondary mechanism: several reports describe downregulation of BST-2 from the cell surface without significant depletion of intracellular levels.[57,60-62] Degradation can be observed when Vpu and BST-2 are expressed at high levels in cells by transient transfection,[58,133] and this mechanism appears to be substantial in at least one of the primary HIV-1 target cells—macrophages, which express high endogenous levels of BST-2.[60,134] The Vpu-induced degradation of BST-2 in HeLa cells and T lymphocytes is not as pronounced and varies between reports.[57,59-61,133] The variability of these results might be caused by differences in the BST-2 species measured: immature (high-mannose) and mature (fully glycosylated) forms of BST-2 are affected differently by Vpu in a manner that relates partly to whether BST-2 is endogenously expressed or is expressed by transient transfection.[133] Specifically, immature forms, which predominate when BST-2 is expressed by transient transfection,

are directed by Vpu to an ERAD-related, proteasomal degradation pathway. In contrast, the transport of mature forms, which predominate when BST-2 is endogenously and constitutively expressed, appears blocked in post-ER membranes by Vpu, and these forms might be degraded in lysosomes by default. These differences also complicate the analysis of the DSGxxS motif and the role of ubiquitination in Vpu activity with respect to BST-2. Potential acceptor sites for ubiquitin in the BST-2 CD have been studied toward the goal of enlightening the debate regarding BST-2 degradation. Mutation of lysine residues (classic ubiquitin acceptors) in the BST-2 CD (KK18,21RR) uncouples the degradation of BST-2 from cell surface downregulation and from the counteraction of restricted virion release; that is, degradation is prevented but Vpu nevertheless reduces the levels of BST-2 at the cell surface and enhances virion release.[62,135] On the other hand, mutation of serines and threonines (of an STS sequence), which are alternative ubiquitin acceptors in the BST-2 CD, renders BST-2 much less efficiently downregulated from the cell surface by Vpu and impairs the ability of Vpu to enhance virion release, although these effects do not correlate with the total cellular levels of BST-2.[63] As noted below, these data suggest that ubiquitin mediated trafficking within the endosomal system might be modulated by Vpu and that this need not lead to protein degradation. In contrast to BST-2, the downregulation of CD4 by Vpu appears exclusively linked to ER retention, extraction from the ER, ubiquitination of the CD of CD4, and the degradation of CD4 by the proteasome.[51]

Lysosomal versus proteosomal degradation. Whether Vpu-induced degradation of BST-2, when it happens, occurs in lysosomes or proteosomes should provide a clue to deciphering the specific trafficking defect(s) caused by Vpu. The use of proteosomal and lysosomal inhibitors in attempts to rescue BST-2 from Vpu-induced degradation or downregulation from the cell surface yielded a variety of conclusions.[57-59,132,135] Although prolonged exposure of cells to proteasome inhibitors blocks the Vpu-induced degradation of BST-2 and inhibits the downregulation of BST-2 from the cell surface,[132] these results arguably reflect ubiquitin depletion and inhibition of ubiquitin-mediated trafficking rather than implicating proteasomal degradation as Vpu's primary mechanism of action.[57] In support of a trafficking defect, Vpu mis-localizes BST-2 from the plasma membrane and various endosomes to a cathepsin-D-positive compartment, when expressed in simian cells, suggesting a lysosomal degradation mechanism.[136] However, in HeLa cells, Vpu does not seem to mis-direct BST-2 to lysosomes but rather redistributes it to perinuclear endosomes including the TGN.[59,61,135,137] Nevertheless, consistent with lysosomal degradation or at least directional transport down the endosomal pH gradient, bafilomycin A, an inhibitor of lysosomal acidification, reduces Vpu-mediated downregulation of BST-2 from the cell surface,[57] and conconamycin A rescues Vpu-induced degradation of BST-2.[59] Moreover, several lines of investigation support a post-ER mechanism of Vpu action with respect to BST-2.[104,133] First, when Vpu is restricted to the ER by appending a specific retention signal to its C-terminus, it is unable to downregulate BST-2 but retains substantial activity with respect to CD4.[104] Second, downregulation of BST-2 from the surface of HeLa cells occurs much faster (a 6-fold reduction within 6 h) than it would if it were based exclusively on the degradation (or retention) of BST-2 in the ER: when the egress of BST-2 from the ER is blocked using brefeldin A (BFA), cell surface levels decrease by only 2-fold after 8 h.[104] Third, in the case of BST-2 expressed endogenously in T cells, treatment with BFA does not enable Vpu to accelerate the degradation of ER-associated immature forms.[133] Thus, a post-ER mechanism involving altered trafficking, lysosomal degradation, or both seems to be involved in the Vpu-dependent counteraction of BST-2, at least when BST-2 is constitutively expressed. Nevertheless, as noted above, ER-associated degradation can occur when BST-2 is expressed exogenously and abundantly by transient transfection of cells such as HEK-293T, as determined by pulse-chase analysis of immature BST-2 species. This effect is presumably caused by fast accumulation of de novo synthesized BST-2, which triggers ER degradation of immature BST-2 forms.[133] Whether this scenario might take place under physiological conditions, such as in macrophages where the expression of BST-2 is high, or in T cells in which the expression of BST-2 is induced by interferon during the innate immune response, remains to be determined.

Vpu serines 52 and 56 and β-TrCP. The preceding arguments apply to the intriguing role of the interaction between Vpu and the E3 ubiquitin ligase complex substrate adaptor β-TrCP during the downregulation of BST-2. Mutating the key serines in Vpu required for this interaction (residues 52 and 56; "Vpu2/6" mutant) partially impairs the downregulation of BST-2 from the cell surface and the enhancement of virion release, at least when BST-2 is expressed at relatively high levels, such as in HeLa cells, macrophages, and HEK 293T cells following transient transfection.[54,57-59,101,134,136] This partial effect contrasts strikingly with the case of CD4 downregulation, for which Vpu2/6 is completely inactive. Interestingly, while CD1d and NTB-A are downregulated from the cell surface, their intracellular levels are not decreased in the presence of Vpu,[64,65] and Vpu2/6 displays a wild-type-like phenotype with respect to the downregulation of NTB-A. Although one might speculate that the extent of the Vpu2/6 phenotype with respect to BST-2 simply reflects the extent of degradation, as discussed above this degradation is not necessarily ER-associated. Therefore, the Vpu-mediated downregulation of BST-2 might rely instead on β-TrCP-based ubiquitination followed by lysosomal degradation, as in the case of the ligand-induced internalization and degradation of IFNAR1, the type 1 interferon receptor.[138] Moreover, the Vpu-β-TrCP interaction might be required not only for degradation but also for the trafficking effects that presumably underlie the robust downregulation of cell surface BST-2 that can be observed despite minimal depletion of total cellular protein.[57] These observations imply that Vpu-β-TrCP complex formation and substrate ubiquitination are involved, at least in the case of BST-2, in the regulation of endosomal trafficking. In contrast, β-TrCP was recently proposed as dispensable for the downregulation of BST-2 based on a failure of RNA interference targeting β-TrCP to block this Vpu activity under conditions that were sufficient to impair the downregulation of CD4.[54] However, these data should be considered

cautiously, because a β-TrCP mutant lacking the F-box domain (ΔF-box β-TrCP) required for interaction with the E3 ligase complex inhibits Vpu activity. Since over-expression of the wild-type β-TrCP does not inhibit Vpu, the notion that ΔF-box β-TrCP acts simply as an inhibitory ligand of the Vpu CD seems less likely. Nevertheless, this report raised the formal possibility that serines 52 and 56 in the CD of Vpu might be required for interaction with a factor other than β-TrCP that is important for the downregulation of BST-2 (**Fig. 2**).

Specific trafficking defects induced by Vpu. To downregulate BST-2 and other proteins from the cell surface, Vpu must alter their normal trafficking. Vpu does not increase the rate of internalization of any of its known targets (BST-2, CD1d or NTB-A).[57,64,65] Although potential adaptor protein-binding motifs exist in the CD of Vpu, no AP complexes or other trafficking factors have been reported to interact with Vpu so far and mutating the putative AP-binding sequences in the Vpu cytosolic domain does not markedly impair BST-2 downregulation or enhancement of virion release[105,106] (and our unpublished data). Likewise, mutating the AP-binding YxY sequence in the cytosolic domain of BST-2 (reportedly required for internalization in the absence of Vpu) does not impair Vpu-mediated downregulation.[61,135] Nevertheless, components of the clathrin-based internalization machinery—the μ subunit of AP-2 (but not of AP-1 or AP-3), the clathrin assembly protein AP180, and the vesicle "pinch-ase" dynamin 2—are each required for optimal downregulation of BST-2 by Vpu[57,136] (and our unpublished data). Currently, the precise role of the internalization machinery in the downregulation of BST-2 by Vpu remains to be deciphered. However, one possibility is that the effects noted above are indirect; for example, the actual effect of Vpu might be a block to the recycling of BST-2 that has been internalized constitutively as discussed below.

While not affecting the internalization rate of BST-2, Vpu does slow the resupply of BST-2 to the PM. This effect appears to involve both newly synthesized BST-2 and BST-2 that has been internalized from the plasma membrane and would otherwise return there via a recycling pathway.[61,139] A Vpu-mediated recycling defect was supported using a flow cytometric assay that measures newly deposited BST-2 at the PM. The inhibition of egress of BST-2 from the ER by brefeldin A had no effect on this assay, which weighed against the possibility that newly synthesized BST-2 contributed substantially to the observed Vpu-effect.[139] A similar flow cytometric approach suggested that Vpu reduces the rate of recycling of CD1d, preventing the return of CD1d from an EEA-1-positive, early endosomal compartment to the PM.[64]

While trapping BST-2 in intracellular compartments, Vpu could consequently or independently re-route BST-2 into the MVB/lysosomal pathway (**Fig. 1**). Indeed, a recent study suggested that the ESCRT machinery, and specifically the formation of a ternary complex between Vpu, BST-2 and the ESCRT 0 component HRS, promotes the Vpu-mediated counteraction of BST-2.[140] This model is consistent with a role for ubiquitination in the downregulation of BST-2 by Vpu. In addition to HRS, the ESCRT components Tsg101 and Vps4 are involved in this process and further link it to lysosomal degradation. Thus, the combination of retention of newly synthesized BST-2 in the TGN, defective recycling, and enhanced trafficking down the MVB/lysosomal pathway appears to underlie the Vpu-mediated downregulation of BST-2. Whether BST-2 that is directed down the MVB/lysosomal pathway includes newly synthesized protein or is exclusively derived from the PM remains to be determined.

Final Remarks

The recent discovery of new host cell factors that are modulated by Vpu (BST-2, CD1d and NTB-A) and the study of the mechanisms underlying these effects have confirmed Vpu as a modifier of intracellular trafficking, a role long ascribed to Nef. Although Vpu and Nef share certain cellular targets such as CD4, the two viral proteins have distinct mechanisms of action: Nef recruits endosomal coat proteins such as AP complexes to its targets whereas Vpu recruits ubiquitination machinery. These differences might reflect a need for the antagonism of specific cellular proteins from specific compartments, such as the Vpu-mediated disruption of the interaction between CD4 and Env in the ER. Nevertheless, the net effect of all the perturbations reviewed here involves the altered expression of plasma membrane proteins. Interestingly, the envelope protein of HIV-2, a virus that lacks Vpu, evolved in certain isolates to downregulate BST-2 from the cell surface, directing the accumulation of BST-2 in a perinuclear compartment.[141] Moreover, mutations in the SIV envelope can lead to the acquisition of a BST-2 modulating activity that compensates for the absence of Nef during viral replication in rhesus macaques.[142] Tyrosine-based AP-binding motifs (Yxxϕ) in the membrane-proximal region of gp41 are essential for the activity of these envelope glycoproteins. Thus, Env is yet another viral protein that is capable of modulating endosomal trafficking to counteract host defenses.

The multifaceted mechanisms underlying the functions of Nef and Vpu present many challenging questions. For example, both Nef and Vpu interact with cellular co-factors even in the absence of their targets. This could in principle cause their mislocalization and/or degradation. Presumably, these viral proteins are either intensely replenished or designed to avoid such suicide inhibition. For example, Vpu itself may not be a substrate for β-TrCP-dependent ubiquitination and degradation, although this is controversial.[53,143-145]

In the case of Vpu, what is the contribution of degradation versus altered trafficking to the downregulation of host targets and what is the physiological meaning of this balance? What is the contribution of altered anterograde transport versus altered recycling? What is the role of clathrin-mediated endocytosis and ubiquitination in these processes? Like Vpu, Nef appears to modulate different proteins by mechanisms that are themselves distinct yet share common elements. How and why does Nef adapt its interactions to meet the needs of different targets? For example, why does the modulation of CD4 by Nef involve AP binding via a canonical ExxxLL motif, yet this motif is dispensable for the modulation of MHC-I?

Perhaps the answers to some of these questions reside within the CDs of the target proteins and of Vpu and Nef. Identifying the components of the endosomal trafficking machinery that interact with these domains will doubtlessly aid in dissecting the mechanisms of altered trafficking. So will determining the sequences within the CDs that are necessary for this modulation. This seems particularly critical to a more complete understanding of Vpu. The complexities of such dissections are exemplified by the distinct roles in degradation and surface downregulation played by lysine, serine, and threonine residues in the cytosolic domain of BST-2, all of which are ubiquitinated by Vpu.[62,63,135] However, the potentially cooperative nature of the interactions between the viral accessory protein, its cellular co-factors, and its target proteins may render the search for novel interactions challenging.

Can our understanding of how Vpu and Nef affect the trafficking of cellular transmembrane proteins be put to clinical use? Since these viral proteins contribute to the virulence of HIV-1 as a pathogen, in principle they represent novel antiretroviral drug targets. Understanding the relevant targets of Vpu and Nef can lead to drug screening assays. Moreover, the structural basis of the ternary interactions between the viral accessory protein, its cellular targets, and its cellular cofactors, might enable rational drug design. Most importantly, both Vpu and Nef are involved in immune evasion, and these functions undoubtedly contribute to the success of HIV-1 as an agent of chronic, persistent infection. We are intrigued by the possibility that the inhibition of Vpu and Nef could empower the host's innate and adaptive immune responses to better clear the infection. In the best case, such empowerment would be sufficient to enable viral clearance and the resolution of infection.

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