Research article

Endophilins interact with Moloney murine leukemia virus Gag and modulate virion production

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Abstract

Background: The retroviral Gag protein is the central player in the process of virion assembly at the plasma membrane, and is sufficient to induce the formation and release of virus-like particles. Recent evidence suggests that Gag may co-opt the host cell's endocytic machinery to facilitate retroviral assembly and release.

Results: A search for novel partners interacting with the Gag protein of the Moloney murine leukemia virus (Mo-MuLV) via the yeast two-hybrid protein-protein interaction assay resulted in the identification of endophilin 2, a component of the machinery involved in clathrin-mediated endocytosis. We demonstrate that endophilin interacts with the matrix or MA domain of the Gag protein of Mo-MuLV, but not of human immunodeficiency virus, HIV. Both exogenously expressed and endogenous endophilin are incorporated into Mo-MuLV viral particles. Titration experiments suggest that the binding sites for inclusion of endophilin into viral particles are limited and saturable. Knock-down of endophilin with small interfering RNA (siRNA) had no effect on virion production, but overexpression of endophilin and, to a lesser extent, of several fragments of the protein, result in inhibition of Mo-MuLV virion production, but not of HIV virion production.

Conclusions: This study shows that endophilins interact with Mo-MuLV Gag and affect virion production. The findings imply that endophilin is another component of the large complex that is hijacked by retroviruses to promote virion production.

Background

The Gag protein of retroviruses plays a critical role in virion assembly (for reviews, see [1-3]). When expressed in the absence of all the other virus-encoded components, this polyprotein alone is sufficient for inducing virus-like particle formation from the cell. Hence, the Gag protein has been referred to as a "particle-making machine" [4]. The Gag proteins of the mammalian gamma-retroviruses, such as the Moloney murine leukemia virus (Mo-MuLV), are translated on free ribosomes in the cytoplasm and myristylated at the amino terminus before being translocated to the plasma membrane [5]. They assemble into enveloped, spherical structures and are then released from the cell. During and after virion assembly, Gag precursors are cleaved by the viral protease into four structural proteins - termed MA, p12, CA, and NC - to form infectious virions.

The MA domain is the major region involved in targeting Gag to the membrane. The precursor Gag proteins are anchored to the plasma membrane by an amino-terminal myristate and by ionic interactions between an amino-terminal cluster of basic residues in the MA domain and the acidic plasma membrane surface [6-8]. Amino-acid substitutions or deletions in the matrix protein of type 1 human immunodeficieny virus (HIV-1) or Mo-MuLV were found to block membrane association [9-12] or to redirect virus assembly to cytoplasmic vacuoles (multivesicular bodies, MVBs). These observations highlight the critical role of MA in intracellular transport of Gag polyproteins to the site of viral assembly. Other mutations in MA have been reported to allow Gag proteins to reach the plasma membrane but to result in Gag accumulation beneath the plasma membrane [13,14]. Slightly curved, electron-dense patches were formed, and no further capsid assembly was observed. These studies provide evidence of involvement of the MA domain in an early step during viral budding.

L domains (for 'late assembly functions') are required for the late stages of viral budding. These domains are located at different regions of Gag in different retroviruses. In Mo-MuLV, Rous sarcoma virus (RSV) and Mason-Pfizer monkey virus (MPMV), the L domains contain a highly conserved PPPY motif (Pro-Pro-Pro-Tyr) and are located between the matrix and capsid domains [15-17]. In lentiviruses, the L domains have distinct motifs - PTAPP in HIV-1 [18,19] and YXXL (in the single-letter amino-acid code where X is any amino acid) in equine infectious anemia virus (EIAV) [20] - and are located at the carboxyl terminus of the Gag protein. Despite the lack of sequence homology, many late domains can be functionally interchanged [21-23].

Recent studies have revealed that a group of cellular proteins of the endocytic/multivesicular pathway are involved in the late stages of viral assembly. Tsg101, a protein involved in vacuolar protein sorting, binds to the PTAPP motif and is required for budding of HIV-1 and Ebola virus [24-27]. Similarly, the WW domains of members of the Nedd4-like family of ubiquitin protein ligases interact with the PPPY motif and play roles in the release of viral particles [28,29]. EIAV utilizes the YXXL motif within its Gag to recruit AP-2, a component of the endocytic machinery, and possibly other components such as AIP-1/ALIX, to promote virion assembly and release [20].

To understand further how retroviruses such as Mo-MuLV recruit host cellular factors to promote virion production, we performed a two-hybrid screen of a mouse T-lymphoma cDNA library using a murine viral Gag as 'bait', and identified endophilin 2 as a Gag-interacting partner. Endophilins are involved in the formation of endocytic vesicles from the early onset of budding until fission [30,31]. In this study, we describe characterization of the Gag-endophilin association and its potential role in virion production.

Results
Identification of endophilin 2 as a Gag-interacting protein

The yeast two-hybrid system was used to search for proteins interacting with murine Gag. The Gag protein of the murine AIDS (MAIDS) defective virus is responsible for its pathogenesis, a hyperplasia and immunodeficiency disease [32]. The *gag* gene product of one isolate, BM5def Gag, closely resembles Mo-MuLV Gag in the MA, capsid and NC regions but contains a highly divergent p12 region [33]. A mouse T-lymphoma cDNA library was screened in a two-hybrid assay with the full-length BM5def Gag as bait to identify potential cellular binding partners. From 150,000 yeast transformants screened, 31 positive clones were isolated. On the basis of sequence similarity, ten of the cDNAs encode overlapping portions of the mouse homolog of human endophilin 2 [34] (also known as SH3P8 [35], SH3GL1 [36], and EEN [37]).

Endophilins are evolutionarily conserved proteins involved in the formation of endocytic vesicles [31]. All family members contain an amino-terminal coiled-coil domain, a variable region and a carboxy-terminal SH3 domain. Members of the two major subgroups in the endophilin family, A and B, are only about 20% identical to each other. Endophilin A associates with the cytoplasmic surface of membranes [38], while endophilin B appears to operate at the endoplasmic reticulum and the Golgi complex [39]. Endophilin A has three members in mammals - 1, 2 and 3 - that are about 70% identical to each other at the amino-acid level.

Endophilin 2 was tested for its interaction with Mo-MuLV Gag in the yeast two-hybrid system. The full-length human endophilin 2 was fused to the carboxyl terminus of the Gal4 activation domain (Gal4-AD), and the complete Mo-MuLV Gag and fragments of the protein were fused to the carboxyl terminus of LexA [40]. The yeast two-hybrid strain CTY-5d was cotransformed with plasmids encoding Gal4AD-endophilin 2 and the various LexA-Gag derivatives, and the strength of interaction between the fusion proteins was monitored by staining yeast colonies with X-gal to visualize β-galactosidase activity (Figure 1a). Gag interacted strongly with endophilin 2. Only fusions containing full-length Gag or the MA domain of Gag (Δ6 and MA; see Figure 1a) inter-acted strongly; a large fragment retaining the carboxy-termi-nal half of MA (Δ8) displayed a weak interaction. Other fragments (p12, p12-CA or CA) showed no activity. No blue color developed in yeast cells transformed with DNAs encoding LexA-Gag derivatives plus an empty Gal4AD vector, nor with DNAs encoding Gal4AD-endophilin 2 plus an empty LexA vector, indicating no activation by the fusion proteins themselves. Quantitative β-galactosidase enzyme assays of yeast cultures expressing the various constructs were used to obtain better estimates of the strengths of the interactions (Figure 1a). In agreement with the filter-lift assays, constructs containing MA showed the strongest reporter gene expression. This experiment suggests that the major region responsible for Gag-endophilin interaction lies within the MA domain.

Identifying binding domains in endophilin 2 for Mo-MuLV Gag using the yeast two-hybrid system

To determine the region in endophilin sufficient for binding to Mo-MuLV Gag, amino- or carboxy-terminal fragments of endophilin 2 were fused to the activation domain in Gal4-AD (Figure 1b) and were tested for their interactions with a LexA-Mo-MuLV Gag fusion. Two fragments, ΔSH3 and V+SH3, displayed only weak interaction with Mo-MuLV Gag. Removal of additional portions of endophilin 2 almost completely abrogated the interaction. These results suggest that the intact endophilin, including both amino terminus and SH3 domains, is required for the strongest interaction with Mo-MuLV Gag. No single region could be identified as sufficient for strong binding. Several fragments showed weak binding, significantly above the background level seen with controls.

Interactions between endophilin 2 and other retroviral Gags in the yeast two-hybrid system

To evaluate whether the interaction is conserved among other retroviruses, the interaction of endophilin 2 with multiple Gag polyproteins, including those of RSV, HIV-1, MPMV and simian immunodeficiency virus (SIV), were examined with the yeast two-hybrid system [40-42]. Plasmids encoding
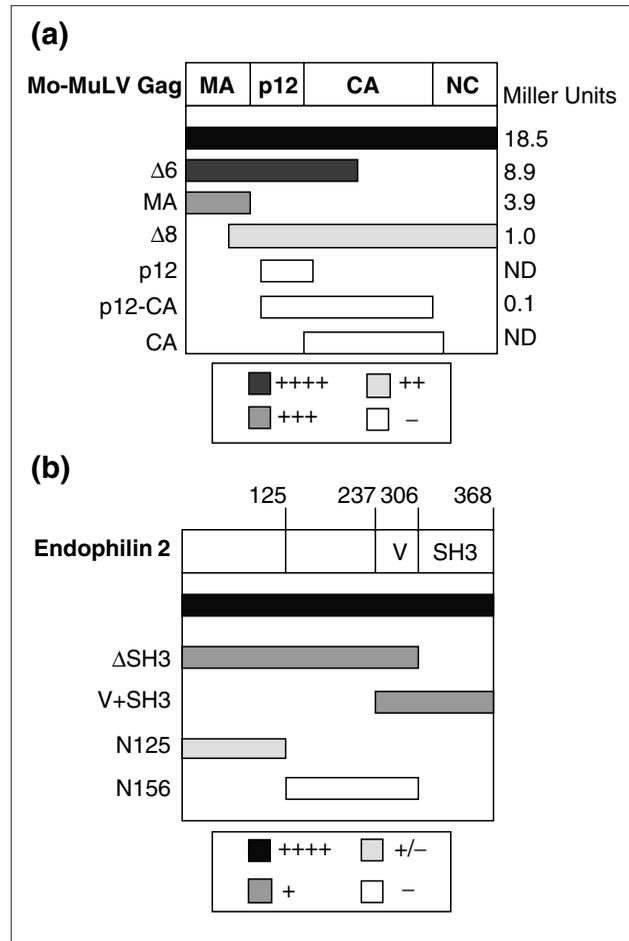

Figure 1
Mapping the binding domains in Mo-MuLV Gag and human endophilin 2 using the yeast two-hybrid system. Yeast strain CTY 10-5d was transformed with different combinations of DNAs encoding the fusion proteins. Fragments of Mo-MuLV Gag were fused to the DNA-binding domain of pSH2LexA, whereas endophilin 2 was fused to the activation domain of pGADNOT. **(a)** Domains in Mo-MuLV Gag assayed for binding to endophilin 2. **(b)** Domains in endophilin assayed for binding to Mo-MuLV Gag. The scoring of β-galactosidase activity of yeast colonies is as follows: -, no blue color after 24 h in reaction; +/-, blue after 8 h; +, blue after 2 h; ++, blue between 30 min and 2 h; +++, blue in approximately 30 min; ++++, blue between 15 and 30 min. All constructs tested negative for self-activation. Quantitation of β-galactosidase levels was as described previously [71].

Gal4AD-endophilin 2 and LexA-RSV Gag were introduced into yeast strain CTY-5d, and plasmids encoding Gal4AD-endophilin 2 and Gal4 binding domain (Gal4BD) coupled to Gag from HIV-1 or MPMV or SIV were introduced into yeast strain GGY::174. The strength of the interaction between Gag and endophilin fusions was assessed by X-gal staining of yeast colonies for β-galactosidase activity (Table 1). Endophilin 2 interacted with RSV Gag but not any of the

other Gags tested. As previously reported, all Gags displayed strong interactions with themselves. Endophilin 2 also interacted strongly with itself in the yeast two-hybrid system, suggesting that it was capable of dimerization. These results indicate that there is a specific interaction between endophilin 2 and some, but not all, retroviral Gags.

Mo-MuLV Gag interacts with another endophilin family member

To determine whether the interaction of Mo-MuLV Gag with endophilin 2 depended on the endophilin's species of origin, plasmids expressing either human or rat endophilin 2 were introduced into yeast along with Gag-expressing plasmids. Both endophilins interacted strongly and equally with Mo-MuLV Gag (Table 2). To test whether the Gag-endophilin interaction is common to another member of the endophilin family, Mo-MuLV Gag was tested for its interaction with rat endophilin 1 in the two-hybrid system. A plasmid encoding rat endophilin 1 fused to the carboxyl terminus of Gal4AD was cotransformed into yeast strain CTY10-5d with a plasmid encoding LexA Mo-MuLV Gag. The interaction of Gag with endophilin 1 was practically as strong as with endophilin 2 (Table 2).

Endophilin 2 binds to Mo-MuLV Gag *in vitro*

To confirm and extend the results with the yeast two-hybrid system, the binding of endophilin 2 to Mo-MuLV Gag *in vitro* was assessed by measuring the interaction of endophilin 2 or its amino-terminal fragments, all expressed as glutathione-S-transferase (GST) fusions, with native Gag produced by a chronically Mo-MuLV-infected NIH 3T3 cell

Table 1

Interactions between endophilin 2 and retroviral Gag proteins in the yeast two-hybrid system

pSH2-LexA fusion	pGADNOT-AD fusion	
	Endophilin 2	Gag
Mo-MuLV Pr65 Gag	+++	++++
RSV Pr65 Gag	++/+	++++
MPMV Pr76 Gag	-	++
SIV Pr57 Gag	-	+++
HIV-1 Pr55 Gag	-	++++
Human endophilin 2	++++	

The various Gag proteins were fused to the DNA-binding domain, and human endophilin 2 was fused to the activation domain. All constructs tested negative for self-activation. Symbols: -, no blue color after 24 h in reaction; +, yeast turn blue after 2 h; ++, blue develops between 30 min and 2 h; +++, blue in approximately 30 min; ++++, blue between 15 and 30 min.

Table 2

Mo-MuLV Gag interacts with another endophilin family member in the yeast two-hybrid system

pGADNOT-AD fusion	pSH2-LexA fusion	
	Mo-MuLV Gag	pSH2-1
Rat endophilin 1	+++	-
Rat endophilin 2	+++	-
Human endophilin 2	+++	-
pGADNOT	-	-

Symbols: -, no blue color after 24 h in reaction; +++, yeast turn blue in approximately 30 min.

line. GST or GST-endophilin fusion proteins were expressed in bacteria, extracts were prepared, and the proteins were resolved by 12% SDS gel electrophoresis. Coomassie Blue staining of the gel verified that the GST fusions were expressed (Figure 2a). Cell lysates from Mo-MuLV-infected cells were prepared and then mixed with bacterial cell lysates containing either GST or GST-fusion proteins; cell lysates from naïve NIH 3T3 cells that did not express Gag were used as a negative control. Glutathione-Sepharose beads were added to the lysate mixture, and the beads were subsequently washed with binding buffer and resuspended in SDS sample buffer.

Proteins eluted from beads were analyzed by western blotting with an anti-capsid antibody. We found that Mo-MuLV Gag was captured only by GST-endophilin-2 beads, but not by GST, GST-N125, or GST-ΔSH3 beads (Figure 2b; compare lane 5 with lanes 2, 3 and 4). Reprobing the same blot with anti-GST antiserum showed that all the GST fusion proteins were successfully bound to the beads and recovered, and so were available for the interaction (data not shown). Gag was detected only in Mo-MuLV-infected NIH 3T3 cell lysates, and not in uninfected NIH 3T3 cell lysates (Figure 2b; lane 5 versus lane 9), indicating that the Gag antiserum did not cross-react with the GST fusion proteins themselves or with any other proteins on the beads. Although the levels of bound Gag proteins were low, binding to full-length endophilin was readily detectable in repeated experiments. Binding of Gag to any of the fragments was too low for detection. These results demonstrate that endophilin 2 and Mo-MuLV Gag can interact *in vitro*.

Exogenously expressed endophilin 2 associates with Mo-MuLV virion particles

To monitor the interaction between endophilin and Mo-MuLV Gag *in vivo*, we investigated whether exogenously expressed endophilin 2 could be incorporated into virion
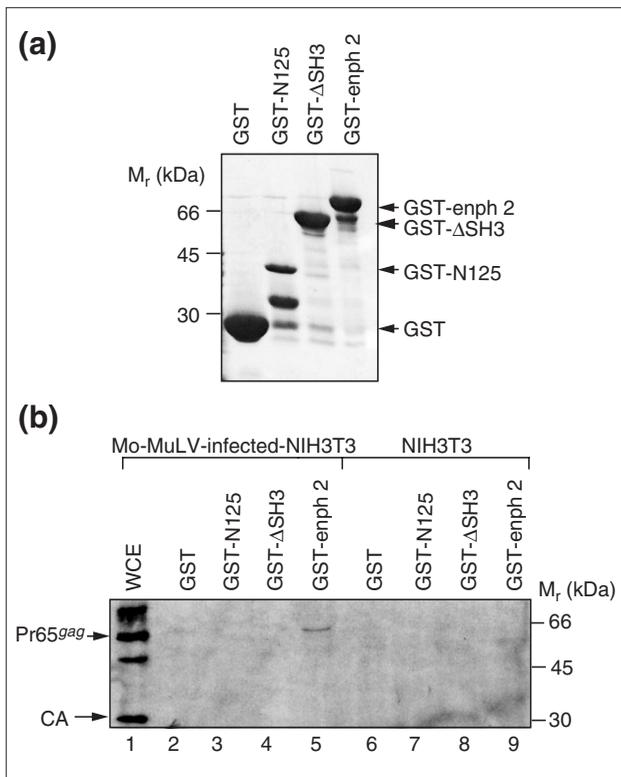
Figure 2
Endophilin 2 binds to Mo-MuLV Gag *in vitro*. **(a)** Bacterial lysates expressing either GST, GST-N125 or GST-ΔSH3 fragments of endophilin or GST-endophilin 2 (full-length; 'enph 2' on this and subsequent figures) were resolved by electrophoresis on 12% SDS gels, and the gel was stained with Coomassie Brilliant Blue. **(b)** Bacterial lysates expressing GST fusions were mixed with mammalian cell lysates either from Mo-MuLV chronically infected NIH 3T3 or from naïve NIH 3T3 cells. Proteins in the cell lysates that bound to GST fusion proteins were recovered with glutathione-Sepharose beads. Beads were then boiled in SDS sample buffer and the proteins were resolved by electrophoresis on 12% gels and analyzed by western blotting with an anti-capsid antibody.

particles. We transfected 293T cells with an expression vector encoding amino-terminal HA-epitope-tagged endophilin 2, either alone or together with a wild-type Mo-MuLV proviral DNA. The culture medium was harvested 48 h post-transfection and virions were purified by sedimentation through 25/45% sucrose step gradients. Virions were collected from the interface of the sucrose gradient, and virion proteins in pellets were solubilized in SDS sample buffer, resolved by gel electrophoresis and analyzed by western blotting. Plasmid DNA encoding an irrelevant HA-tagged protein, annexin II light chain (HA-p11), was used as a negative control. Plasmid DNA encoding an HA-tagged fragment of nucleolin (HA-nuc212), known to be efficiently incorporated into virion particles, served as a positive control [43].

Both the precursor Pr65 Gag in the cell lysate and capsid in virions were detected after transfection of a proviral DNA (Figure 3a,c; lanes 2, 4, 6 and 8). HA-endophilin 2 was clearly detected in particles purified from cells expressing viral proteins (Figure 3d; lane 8), but not from cells in which no Gag was expressed (Figure 3d; lane 7). As anticipated, we observed no HA-p11 in particles (Figure 3d; lane 4 versus lane 3), while substantial levels of HA-nuc212 were recovered in virions (Figure 3d; lane 6 versus lane 5). This experiment suggests that HA-tagged endophilin 2 can specifically associate with Mo-MuLV virion particles.

To obtain an estimate of the proportion of intracellular endophilin 2 that is incorporated into virions, serial dilutions of cell lysates were prepared and compared with virion lysates by analysis on the same western blots. These experiments suggest that virions in the culture medium contain approximately 0.1-0.2% of the HA-endophilin 2 present intracellularly (Figure 3e). This fraction is as much as 100 times lower than the corresponding fraction of a positive control protein, HA-Nuc212, which is very efficiently incorporated into virions. While low, the fraction for endophilin 2 is at least 10 times higher than that for the negative control protein (Figure 3f; less than 0.01%).

To further verify the association of endophilin 2 with virion particles, the preparations were analyzed on linear sucrose gradients. Culture medium was harvested from cells cotransfected with a plasmid encoding HA-endophilin 2 and a proviral DNA, and virions were purified on a 25/45% sucrose step gradient as before. The purified virions were then applied to a 20-60% linear sucrose gradient (Figure 4a). Fractions were collected, and proteins were precipitated by trichloroacetic acid (TCA) and analyzed by western blotting with anti-capsid antibody and anti-HA antibody (Figure 4b,c). A major peak of HA-endophilin, migrating as a doublet of proteins at the expected molecular weight, was detected at a density of about 1.12 g/ml, and comigrating with capsid in fractions 9, 10, and 11. We do not know the origin of the doublet of proteins, though the faster-migrating one of the pair of bands comigrates with the single species detected in the cell (data not shown). A smaller amount of endophilin was also recovered at the top of the gradient (fractions 16 and 17) along with the bulk membrane fraction and low molecular weight proteins that do not enter the gradient. Taken together, these results show that endophilin 2 and Gag associate *in vivo* and copurify in virion particles.

Exogenously expressed endophilin 2 is protected from proteases within Mo-MuLV virion particles
Endophilin 2 could associate with virion particles simply as a contaminant in copurifying microvesicles, or through
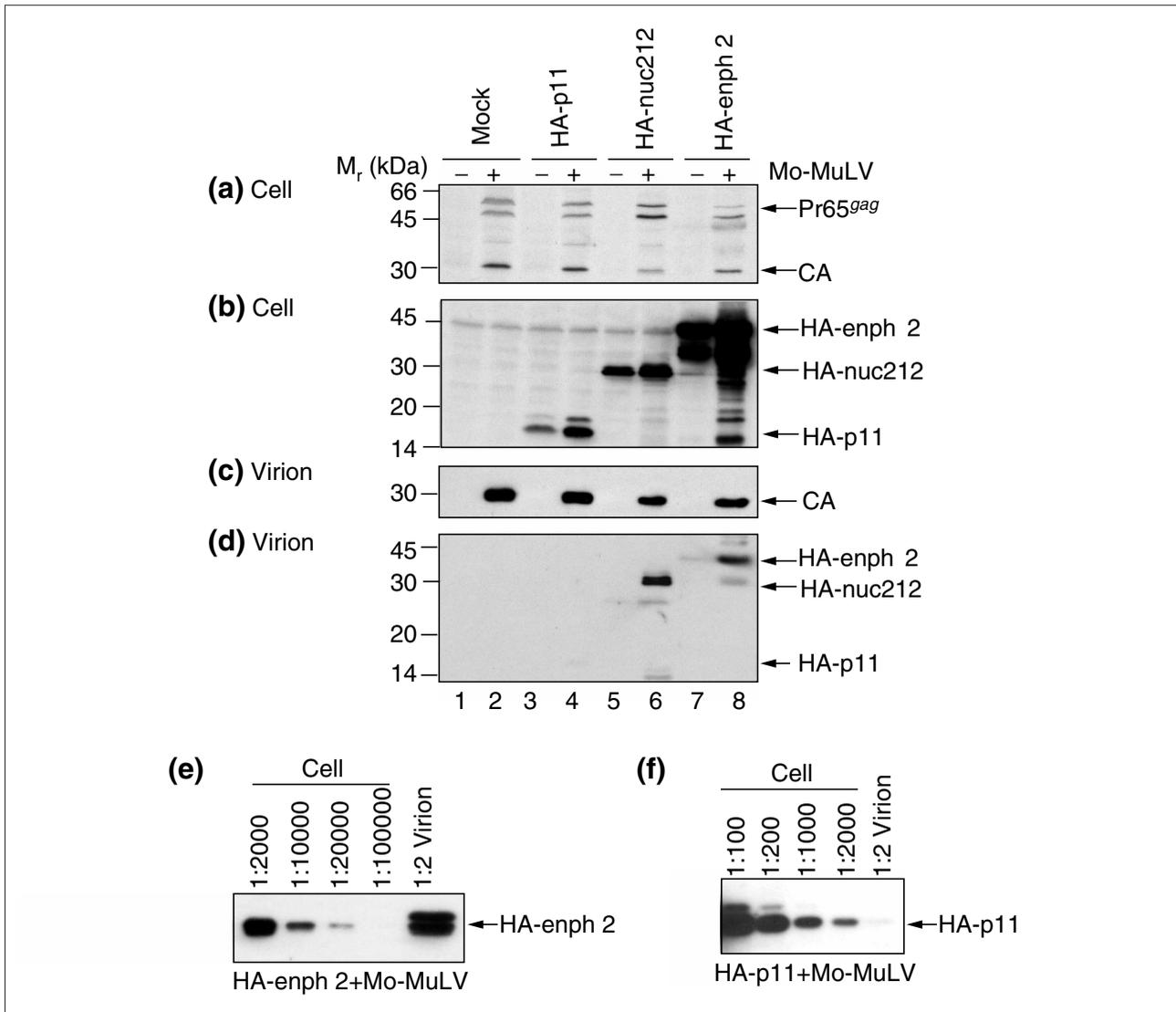
Figure 3
Incorporation of HA-endophilin 2 into Mo-MuLV virions. The 293T cells were transiently transfected with 2 µg of plasmid encoding HA-tagged endophilin 2, either alone or together with 10 µg of proviral DNA, as indicated. The proteins in cell lysates and purified virions were analyzed by western blotting with **(a,c)** anti-capsid and **(b,d)** anti-HA antibodies. Cells were transfected with plasmids expressing **(e)** HA-p11 (annexin II light chain) or **(f)** HA-endophilin 2 along with Mo-MuLV DNA, and serial dilutions of cell lysates and virion proteins were analyzed by western blot with anti-HA antisera.

an association with the outer surface of the budding virions. Nonspecifically associated proteins are sensitive to digestion by subtilisin, while proteins in the virion particles are protected from digestion by the virion envelope [44]. To test whether the copurified endophilin is present inside the viral particles, virion particles purified from culture medium by step gradient were subjected to digestion with increasing amounts of subtilisin. Digested virions were then repurified through a 25% sucrose cushion and their protein components were analyzed by western blotting (Figure 5a). Although envelope proteins on the virion surface were degraded by treatment of the virions with low levels of protease, the capsid and HA-endophilin proteins were protected even at very high concentration of protease. Permeabilization of virion particles with 0.2% NP-40 abolished the protection of capsid and endophilin, and these proteins were then degraded even at low concentration of protease (Figure 5b). This experiment
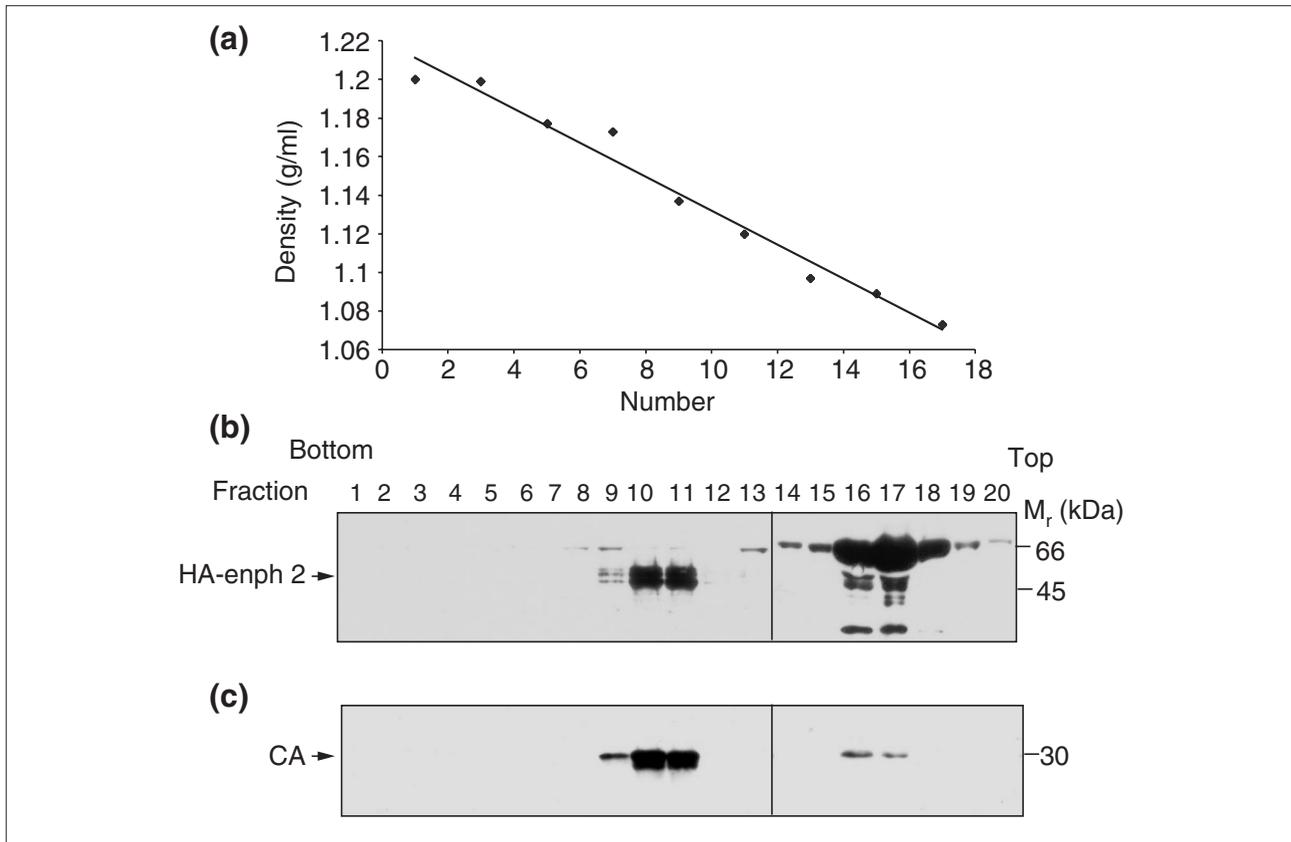
Figure 4
Copurification of HA-endophilin 2 with virions. The 293T cells were cotransfected with 2 μg of plasmid encoding HA-endophilin 2 and 10 μg of proviral DNA. Virion particles purified through a 25/45% sucrose gradient were reloaded on a 20-60% linear sucrose gradient, and 20 fractions were collected. **(a)** A plot of the gradient density. **(b,c)** Proteins in these fractions were precipitated with TCA and analyzed by western blotting with (b) anti-HA and (c) anti-capsid antibodies. The virions migrate near the middle of the gradient and are marked by the comigration of CA and a doublet of endophilin proteins (fractions 9-11). A small amount of endophilin is also found at the top of the gradient (fractions 16-19). The bulk of the membrane-associated and low molecular weight proteins remains at the top of the gradient; the huge amount of protein in these fractions cause distortions in the mobility of the proteins in these lanes. High-molecular-weight proteins at the top of the gradient are also recognized by weak nonspecific reactivity in the anti-HA antibody.

strongly suggests that HA-endophilin is incorporated inside the virions.

The incorporation of exogenously expressed endophilin 2 is saturable

To characterize the association further, we examined the level of incorporation of endophilins into virions with increasing levels of expression in the producer cells. The 293T cells were transfected with increasing amounts of plasmid DNA encoding HA-endophilin in the presence of a constant level of proviral DNA. The culture medium was harvested and purified on a 25%/45% step gradient, and proteins in the virion particles and in cell lysates were analyzed by western blotting with an anti-HA and an anti-capsid antibody. The levels of incorporated endophilin 2

inside the virions quickly reached a plateau, and no higher levels were found even with dramatically increasing amounts of endophilin 2 expressed inside the cells (Figure 6). This experiment suggests that the binding sites for endophilin inside the virions are limited and saturable. Furthermore, it is unlikely that the recovery of endophilin 2 in the virion particles can be attributed to nonspecific contamination by retention of cellular membrane components.

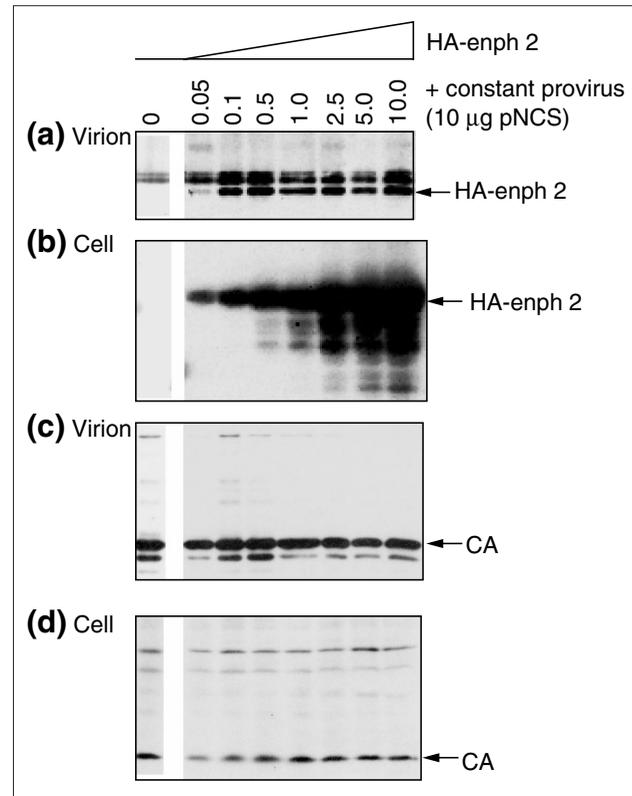## Incorporation of endogenous endophilin 2 and other endocytic proteins into Mo-MuLV virion particles

To investigate whether endogenous endophilin 2 is incorporated into virion particles, 293T cells were either mock-transfected or transfected with a Mo-MuLV proviral DNA. Culture supernatants were collected and virions were
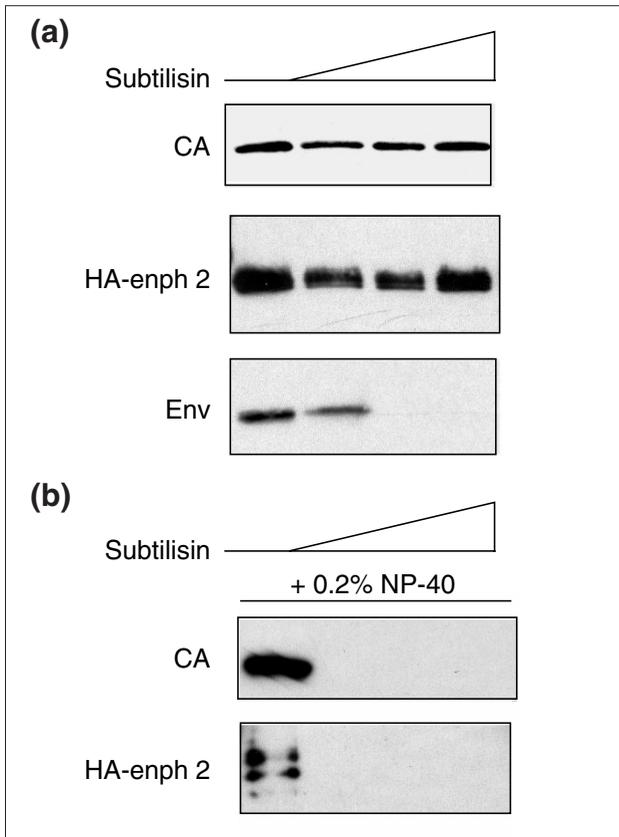
Figure 5
HA-endophilin 2 is incorporated inside Mo-MuLV virions. Equal amounts of purified virion particles were subjected to subtilisin digestion at 0, 1, 10 or 100 μg/ml. Protease inhibitors PMSF and aprotinin were subsequently added to terminate the digestion.
(a) Virion particles after digestion overnight at room temperature were sedimented through a 25% sucrose cushion, and the proteins in the pellets were analyzed by western blotting with anti-HA, anti-capsid and anti-p15E envelope antibodies. **(b)** Virion particles were subject to subtilisin treatment in the presence of 0.2% NP-40, and then were directly analyzed by western blotting with anti-HA and anti-capsid antibodies.

Figure 6
Levels of endophilin 2 incorporation are saturable. A plasmid encoding HA-endophilin 2 was cotransfected at a level of 0, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0 μg with 10 μg of Mo-MuLV proviral DNA, pNCS. The proteins in **(a,c)** viral particles and **(b,d)** cell lysates were analyzed by western blotting with (a,b) anti-HA and (c,d) anti-capsid antibodies.

purified through 25%/45% sucrose layers. Proteins in the virions were analyzed by western blotting with antibodies specific for various endocytic proteins. Endogenous endophilin 2 was incorporated at significant levels into the virions only from cells expressing Gag, but not when Gag was absent (Figure 7a). Comparison of the levels in the intracellular lysates with the virion lysates on these blots suggests that about 0.7% of the endogenous endophilin 2 is recovered in the virus. Other endocytic components that were substantially detected in the virion particles were a subunit of the AP-2 adaptor complex (α-adaptin; about 0.1-0.2% of intracellular levels) and clathrin (about 2% of intracellular levels; Figure 7b,c). In contrast, dynamin 2, the

major endocytic partner of endophilin, was not detectably incorporated (Figure 7d). Without calibration with standards, it is difficult to estimate the amounts of these molecules per virion. Nevertheless, these results suggest that not every endocytic protein is significantly incorporated into virion particles, and that endophilin is not accidentally incorporated just because of its proximity to the plasma membrane.

Two of these virion-associated proteins were tested for their resistance to protease digestion by subtilisin, as was done previously for exogenously expressed HA-endophilin. Both α-adaptin and clathrin present in the virion preparations were fully protected from proteolysis, under conditions in which the external viral Env protein was fully degraded (Figure 7e,f). Thus, these proteins are not simply bound to the outside of the virions, nor released from cells in association with the microvesicles that are known to contaminate virion preparations [44].
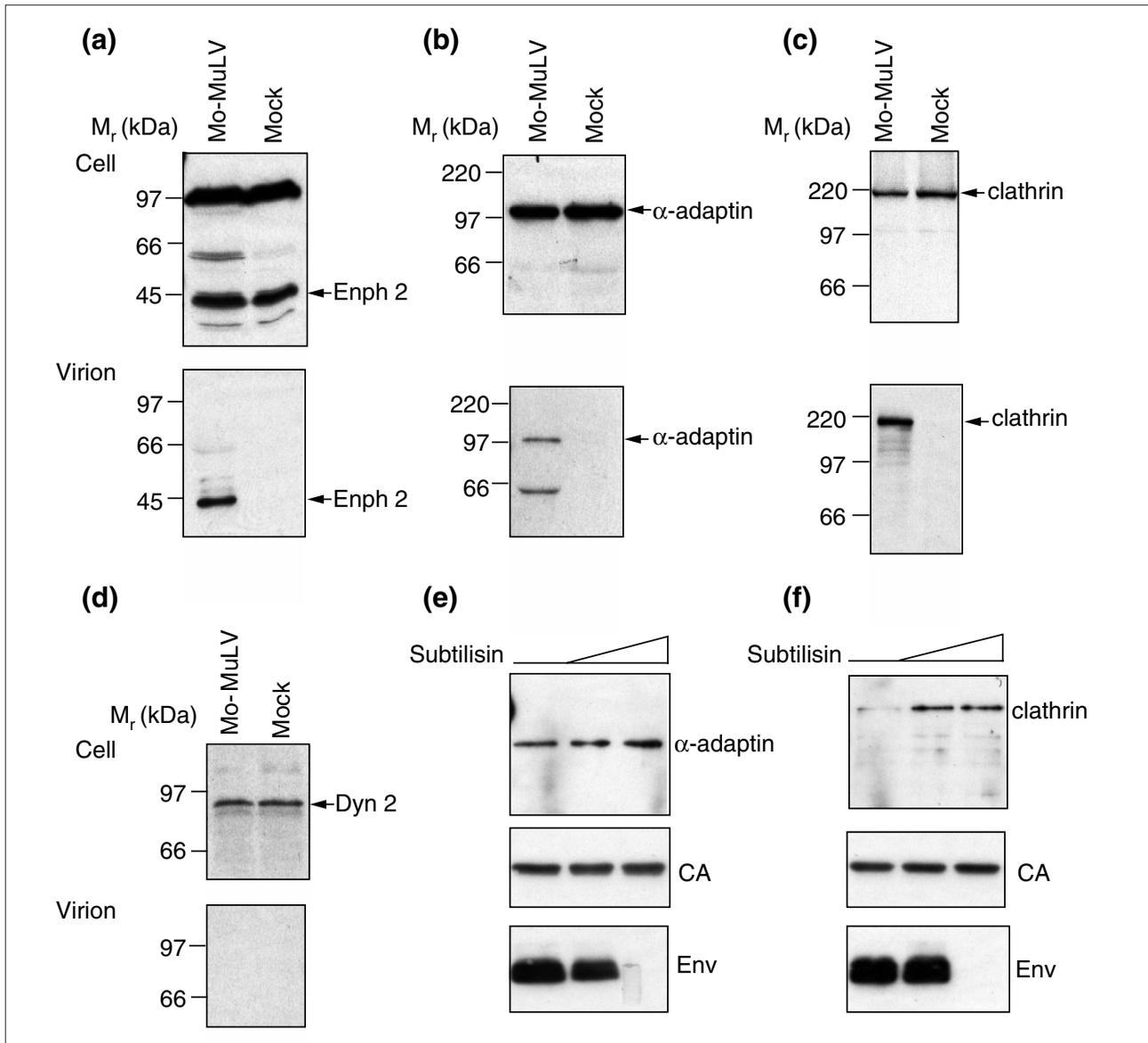
Figure 7
Endogenous endophilin 2 and other endocytic proteins are incorporated into Mo-MuLV virions. The 293T cells were either mock-transfected or transfected with a proviral DNA, pNCS. Equal amounts of cell lysates and virion particles were analyzed by western blotting with various antisera: **(a)** anti-endophilin 2; **(b)** anti-α-adaptin (a subunit of AP-2); **(c)** anti-clathrin; **(d)** anti-dynamin 2. Two of these incorporated proteins were shown to be protected from protease digestion within the virions after treatment with increasing levels of subtilisin: **(e)** α-adaptin and **(f)** clathrin. Under these conditions, the viral envelope protein is digested while the internal CA protein is protected.

Knock-down of endophilin 2 does not inhibit virion production

To evaluate the importance of endophilin 2 in virion production, we examined virus yields in 293T cells depleted of endophilin 2 by using synthetic small interfering (si) RNAs. The 293T cells were transfected twice at 24 h intervals with each of two pairs of synthetic siRNAs that were derived from different regions of the endophilin 2 mRNA sequence. During the second transfection, a Mo-MuLV proviral DNA was cotransfected with the siRNAs. After an additional 24 h post-transfection, culture medium was harvested and virions were purified through a 25% sucrose cushion. Both proteins in cell lysates and in viral particles were analyzed by western blotting (Figure 8a). Endophilin 2 levels were

knocked down to about 20% of normal levels with siRNA1 (Figure 8a; lanes 1 and 4 compared to lane 3) or about 50% with siRNA2 (Figure 8a; compare lanes 2 and 3); but we did not observe any significant change of levels of virion-associated capsid. The reverse transcriptase activity of culture medium displayed at most a two-fold reduction compared to controls (Figure 8b). This experiment suggests that the knock-down of endophilin 2 to these levels has no significant effect on the course of viral production.

Inhibition of virion production by overexpression of fragments of endophilin 2

If endophilins are important for virion production, the overexpression of fragments or of wild-type endophilin 2 could exert dominant-negative effects on virion production. A number of such constructs have been shown to affect endocytosis [45-48]. N125 is a fragment of endophilin 2, similar to the equivalent construct of endophilin 1, which binds to lipsomes and tubulates them *in vitro*; N156 is a fragment with a coiled-coil region; ΔSH3 contains both N125 and N156 regions but lacks the SH3 domain; V+SH3

Figure 8
Knock-down of endogenous endophilin 2 has no effect on viral production. Synthetic siRNAs were transfected twice into 293T cells. A Mo-MuLV proviral DNA pNCA was cotransfected with siRNAs at the second transfection (see text). **(a)** Proteins in cell lysate and virion particles were analyzed by western blotting with anti-endophilin 2 and anti-capsid antibodies. **(b)** Virion production was monitored by assaying reverse-transcriptase activity.

contains both variable and SH3 domains and has previously been shown to interact with dynamin, synaptojanin and amphiphysin [34,38,49]. These fragments were each tagged with an influenza virus HA epitope at the amino terminus. We first examined incorporation of these fragments into virions with low expression in producer cells. We cotransfected 293T cells transiently with a plasmid containing an individual HA-tagged fragment and a proviral DNA at a 1:5 ratio. Culture supernatants were collected 48 h post-transfection and virions were purified through a 25%/45% sucrose step gradient.

Analyzing proteins in cell lysates by western blotting revealed that each fragment was expressed at substantial levels, although some accumulated to higher levels than others (Figure 9a; lanes 1-6). All four of the fragments were incorporated into virions (Figure 9b; lanes 1-6). Serial dilutions of the cell lysates were analyzed together with the virion preparations on the same blots, to allow estimation of the fraction of the intracellular proteins that were recovered in the virions. To examine the potential role of the amino terminus of endophilin 2 in incorporation, a mutant lacking the first 33 amino acids (Δ34) was also tested and found to be equally well incorporated (data not shown). In the case of the full-length HA-endophilin 2, as well as each fragment, approximately 0.1-0.2% of the intracellular protein was found in the virions. No single region of the protein thus seemed to be essential for incorporation; the fragments were incorporated even though they did not show strong direct interaction with Gag in the yeast two-hybrid assay system or *in vitro*. These results suggest that the incorporation could be indirect, either through dimerization with endogenous endophilin or interaction with other cellular proteins that make direct contact with Gag. Alternatively, there may be redundant contacts, or multiple regions of endophilin that can mediate virion incorporation.

We proceeded to test the ability of these fragments to exert a dominant-negative effect on virion production. To do so, we cotransfected 293T cells transiently with a plasmid containing an individual HA-tagged fragment and a proviral DNA (pNCA) at a 5:1 ratio rather than at a 1:5 ratio. Controls included for this experiment were: mock transfections; cotransfection of proviral DNA with an empty expression vector (Figure 10, lane 2); and cotransfection of a reporter plasmid encoding a firefly luciferase to monitor for cytotoxicity (Figure 10, lanes 2-7, and data not shown). Culture medium was collected and virions were purified on a 25%/45% sucrose step gradient. The equivalent amounts of cell lysate and virion released in culture medium were analyzed by western blotting. The fragments were readily detected in transiently transfected cells (Figure 10a, top panel; lanes 2-7). The overall level or stability of the
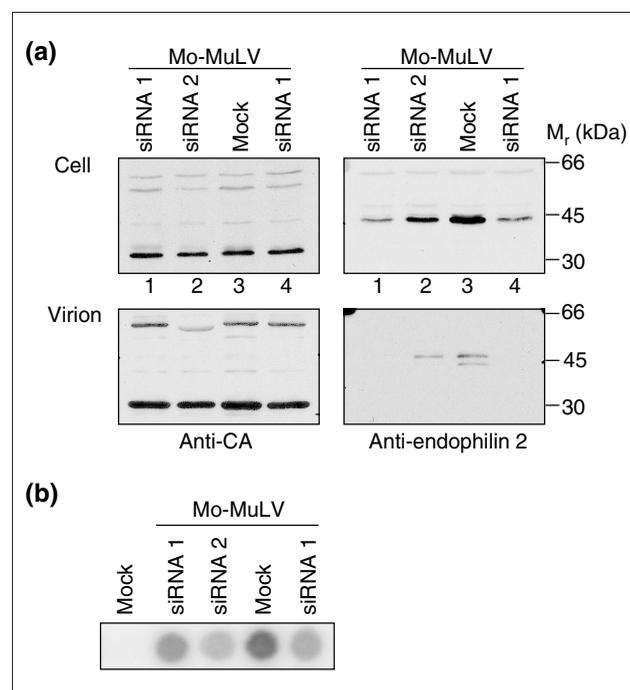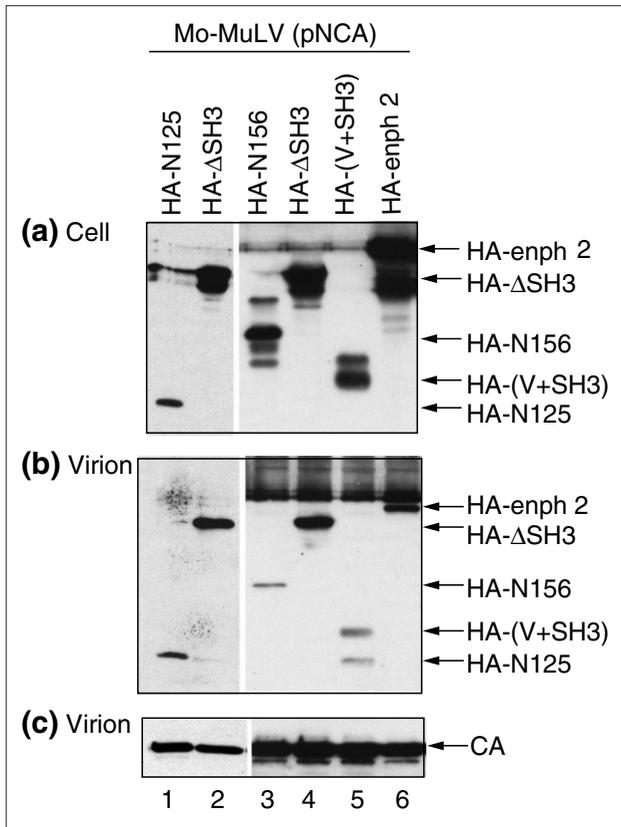
Figure 9
Incorporation of fragments of endophilin 2 into virions. The 293T cells were cotransfected with 1 μg of plasmid encoding HA-tagged endophilin 2 fragments and 5 μg of Mo-MuLV proviral DNA. Proteins in **(a)** cell lysates and **(b,c)** virion particles were analyzed by western blotting with (a,b) anti-HA and (c) anti-capsid antibodies.

To extend these observations, we cotransfected 293T cells with Mo-MuLV proviral construct pNCS and full-length endophilin at increasing DNA ratios (1:0, 1:1, 1:2.5 and 1:5; Figure 10b). We found that endophilin overexpression induced a clear dose-dependent inhibition of Mo-MuLV virion production. At a 1:2.5 ratio, virion production was inhibited 3- to 4-fold; at a 1:5 ratio, more than 10-fold inhibition was observed (Figure 10b; lanes 4 and 5).

We also tested the effect of overexpressing full-length endophilin 2 or fragments of it on HIV-1 virion production. The experiment was carried out the same way as for Mo-MuLV, except an HIV-1 codon-optimized *gag-pol* DNA construct (pCMVgagpolBNkan) was used to generate HIV-1 virus-like particles. Overexpression of full-length endophilin or fragments of it had only a minor effect, if any, on HIV-1 pr55Gag-based virus-like particle production (Figure 10c). The N125 fragment had the only reproducible effect, and this was a marginal one. Taken together, these results suggest that high-level overexpression of endophilin 2 can induce a significant inhibition of virion production for Mo-MuLV, but not for HIV Gag production in 293T cells. The almost complete lack of effect on HIV-1 virion production suggests that the overexpression of these proteins is not causing broad metabolic toxicity or depletion of plasma membrane as the mechanism of blocking Mo-MuLV virion production.

Discussion

In the experiments described here, we have presented evidence that endophilins interact with the matrix or MA domain of the Mo-MuLV Gag protein and may contribute to the process of virion production. We detected an interaction between Mo-MuLV Gag and endophilin 2 in the yeast two-hybrid system, and *in vitro*, and *in vivo*. The analogous interaction was not detected for all retroviral Gags, but was seen for Mo-MuLV and RSV Gags. Further tests showed that exogenously expressed endophilin 2 is associated with virion particles, and is protected within the viral envelope. Several fragments of endophilin 2 were also incorporated into virions; these experiments did not identify any specific domain of endophilin as essential for the process, and it is possible that more than one domain can direct incorporation. The endophilin fragments may have been targeted to virions by dimerization with endogenous endophilin, or by indirect interactions with other proteins. About 0.1-0.2% of the intracellular levels of endophilin were recovered in virus, even for those fragments which bound poorly to Gag in yeast. Titrating the levels of endophilin expression showed that the binding sites for endophilins during virion formation are limited and the level of incorporated protein is saturable. These observations suggest that the presence of endophilins within virion particles is not simply attributable to mass

intracellular Gag precursor protein Pr65 was largely unaffected by overexpression of endophilin 2 or its derivatives or an empty vector control (Figure 10a, middle panel; lanes 2-7), indicating that there were no general cytotoxic effects induced by overexpression of these fragments. Overexpression of full-length endophilin 2 dramatically decreased (approximately 10-fold) the level of virion-associated Gag and capsid detected in the culture medium (Figure 10a, bottom panel; lane 6), as compared to that of the empty vector transfected cells (Figure 10a, bottom panel; lane 2). Overexpression of N125, ΔSH3 or V+SH3 caused modest reduction (around 2- to 3-fold) in the yield of both virion-associated Gag precursors Pr65 and capsid (Figure 10a, bottom panel; lanes 3, 5 and 7). In contrast, we observed that overexpression of N156 at levels comparable to that of the other fragments showed little inhibition of virion-associated Gag and capsid production (Figure 10a, bottom panel; lane 4).
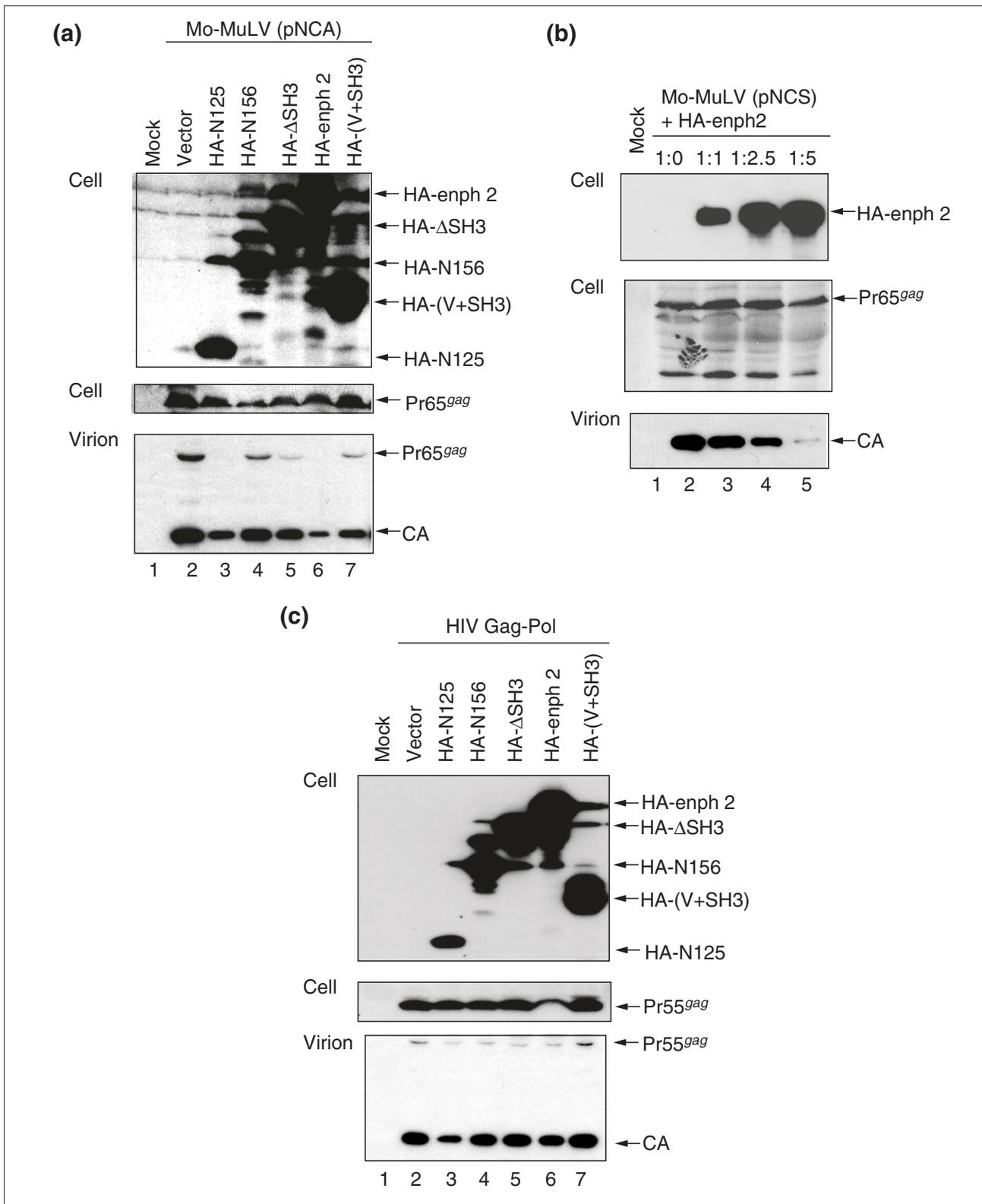
Figure 10 *(see legend on the next page)*

action, but that the incorporation might be mediated through specific contacts, with only a limited number of sites for Gag-endophilin association. It is not clear whether the incorporation *per se* is involved in virion production.

If the interaction is crucial for virion production, we thought it possible that overexpression of full-length or fragments of endophilins might interfere with this process by perturbing the correct stoichiometry of the interaction between endophilin and Gag, or other proteins required for this process. Consistent with this notion, overexpression of full-length endophilin 2 did act in a dominant-negative fashion to significantly reduce Mo-MuLV virion production. The inhibition occurred in a dose-responsive manner. Overexpression of endophilin 2 or N156, a fragment that contains a coiled-coil region, had no effect on the level of expression of Gag precursor or a reporter gene within the cells. Moreover, overexpression of endophilin or its fragments had no or little effect on the production of HIV virus-like particles, correlating with our observation that there is no direct interaction between HIV-1 pr55Gag and endophilin. These data further support the notion that the inhibition of Mo-MuLV virion production we observed is not a nonspecific consequence of overexpression on cell viability or physiology. Rather, overexpression could titrate out Mo-MuLV Gag or other interacting proteins that are specifically required for Mo-MuLV and not HIV production.

We used the siRNA method to knock-down endogenous endophilin 2. This technique has been successfully used to document the requirement of TSG101 in virion assembly [25]. In our case, however, the knock-down of endogenous endophilin 2 had no significant effect on virion yield. One possibility is that the levels of endophilins remaining inside cells after knock-down are sufficient to execute the required functions; indeed, the levels required for virion production may be very low, as only a very small proportion of the intracellular protein is incorporated. The other possibility is that other endophilin family members could compensate for the loss of expression. Indeed, this is very likely because endophilin 1 is expressed in 293T cells (data not shown) and we have shown that it can interact with Gag. It is not clear if the various endophilin family members are fully interchangeable for this or even host functions. We do know that the sequence of the siRNA oligonucleotides used in these experiments is specific to endophilin 2, and would not be able to affect the levels of endophilin 1. We were unable to identify a sequence of suitable length that was an identical match between the two mRNAs.

Retrovirus budding is the topological reverse of endocytosis [50], and membrane curvature changes dramatically from the relatively planar plasma membranes during formation of 100 nm virions. So far it has not been documented whether proteins that modify membrane curvature contribute to retroviral budding. In this study, we have shown that overexpression of N125, a fragment of endophilin that binds liposomes and induces tubules of diameter 20-100 nm [39], or of full-length endophilin, a protein that presumably promotes both positive and negative membrane curvature changes during the formation of endocytic vesicles [48,51], causes a significant reduction of virion production. These observations raise the possibility that our overexpression disrupts normal endophilin functions, and that the binding of lipids and subsequent promotion of changes in plasma membrane curvature could be one of the functions normally exerted by endophilin to assist Mo-MuLV virion formation. It is noteworthy that clathrin, like Gag, was long thought to be sufficient to drive membrane budding by itself. More recently it has become evident that groups of proteins have to work together to bend a biological membrane [52]. Endophilin is not the only protein that can induce membrane curvature. At least three other proteins (dynamin, amphiphysin and epsin) that are involved in clathrin-mediacted endocytosis can independently trigger membrane tubulation *in vitro* [53-55]. These proteins might function at multiple stages during vesicle formation. Morphological analyses indicate that endophilin A is required for clathrin-mediated synaptic vesicle endocytosis at multiple stages, including the following: the early stage of endocytosis, the formation of shallow pits; late stages, with the formation of deeply invaginated, elongated pits; and fission [45,46]. Proteins such as endophilin, which can help generate membrane curvature, might be involved in virion production at several stages, from formation of slightly curved membrane structures to stalk-like structures until viral fission.

Figure 10 *(see figure on the previous page)*
Effects on the release of virion-associated capsid of overexpression of endophilin 2 and its fragments. Plasmid (5 μg) encoding HA-tagged endophilin 2 fragments was transfected into 293T cells with either **(a)** 1 μg of Mo-MuLV proviral DNA (pNCA) or **(c)** 1 μg of HIV-1 codon-optimized *gag-pol* DNA construct CMVgagpolBNkan. In **(b)**, 293T cells were cotransfected with 1μg of Mo-MuLV proviral DNA (pNCS) and endophilin 2 at ratios of 1:0, 1:1, 1:2.5 and 1:5. The total DNA for each transfection was normalized at an amount of 6 μg by addition of empty vector plasmid. Equal amounts of cell lysate and virion particles were analyzed by western blotting with anti-HA (top panels in a, b and c), anti-Mo-MuLV capsid (middle and bottom panels in a and b) or anti-p24 antibodies (c, middle and bottom panels).

Endophilin may act as part of a large complex, and may associate with many other proteins. The SH3 domain of endophilin has been shown to bind to the proline-rich domain of dynamin [38], amphiphysin [34] and synapto-janin, a phosphatidylinositol 5′-phosphatase implicated in synaptic-vesicle uncoating [49]. We found that AP-2 and clathrin, but not dynamin 2, are significantly incorporated into virion particles. The incorporation of any one of the various proteins involved in vesicular trafficking may depend on the behavior of the particular complex in which it resides; one explanation for the lack of incorporation of dynamin 2 is that it may interact with a distinct pool of endophilins, one that does not interact with Gag.

Among the proteins associated with Gag are several other proteins that are implicated in retrovirus budding. Tsg101 is known to interact with the PTAP motif of the L domain of HIV-1 and many other retroviruses, and is required for efficient virion release. Members of the Nedd4 family, involved in endocytosis and recycling of membrane proteins, interact with the PPPY motif of the L domain of many other retro-viruses and play a similar role. Dominant-negative fragments of Nedd4-like family members inhibit the release of viral particles much as we have seen for endophilin [28,29]. These observations suggest that a completely functioning vesicular trafficking pathway is required for retroviruses budding [50,56,57]. Interactions with vesicles may also be involved in earlier stages of trafficking of genomic RNA, Env and Gag to the cell surface [58]; possibly the binding of endophilins to Gag can promote their association with endosomal vesicles.

Very recently, HIV-1 Gag has been shown to associate with the endocytic protein AIP-1/Alix through specific contacts with the Gag p6 domain [59,60]; Alix is known to interact with endophilins [61]. This observation, along with our results, suggests that endophilin could be another component that is hijacked by retroviruses to promote virion production.

Materials and methods
Yeast two-hybrid system
Yeast reporter strains CTY10-5d and GGY::171 [62] were generously provided by R. Sternglanz. The yeast expression vector pSH2-1 [63] encodes an amino-terminal LexA DNA-binding domain (LexADB); pGADNOT encodes a carboxy-terminal Gal4 activation domain (Gal4AD) [64]. Plasmids containing Mo-MuLV, RSV, HIV-1, SIV-1 and MPMV Gag were described previously [40-42]. Plasmids Δ6 and Δ8 are identical to 3′ Δ2355 and 5′ Δ1304, respectively, as described previously [40]. DNAs encoding Mo-MuLV MA, p12, p12-CA and CA were amplified from pNCA [65] by PCR and cloned into plasmid pSH2-1 to generate yeast two-hybrid plasmids.

Yeast two-hybrid library screen
BM5def Gag was cloned into pGBT9 DNA-binding domain vector (Clontech, Palo Alto, USA) and used to screen a V13 T-lymphoma cDNA library [66].

Recombinant proteins
The full-length cDNA encoding human endophilin 2 was obtained from Chi Wai So (University of Hong Kong) [67-69]. DNA encoding human endophilin 2 residues 1-125 (N125), 1-306 (ΔSH3) or full length (1-368) was cloned into pGEX2TKPL, a derivative of pGEX-2TK (Pharmacia, Pis-cataway, USA). GST-fusion proteins were produced in bacteria cells as described previously [43].

Mammalian plasmid DNAs
Mo-MuLV was expressed either from plasmid pNCA [65] or from pNCS, a derivative carrying an SV40 origin of replication in the plasmid backbone. The plasmid pCMVgag-polBNkan (provided by George Pavlakis) contains a Rev-independent, codon-optimized HIV-1 Gag-Pol gene driven by a cytomegalovirus (CMV) promoter. The plasmids encoding hemagglutinin (HA)-tagged nuc212 or p11 were as described [43]. DNA encoding human endophilin 2 residues 1-125 (N125), 125-306 (N156), 1-306 (ΔSH3), 34-368 (Δ34), 268-368 (V+SH3), or full-length 1-368 (enph 2) was cloned into a pcDNA3.1 vector encoding an amino-terminal HA-epitope tag.

Transfection and siRNA
Four 21-nucleotide single-strand RNAs with symmetric 2 nucleotide 3′ (2′-deoxy) thymidine overhangs were ordered from Dharmacon (Lafayette, USA). The sequence of siRNA 1 is: sense, 5′-CACGGUGUCCAAGAUCCGTT-3′; antisense, 5′-ACGGAUCUUGGACACCGUGTT-3′. The sequence of siRNA 2 is: sense, 5′-GUUCGAGGAGUCCAAGGAGTT-3′; antisense, 5′-CUCCUUGGACUCCUCGAACTT-3′. These single-strand RNAs were annealed to produce duplexes.

The 293T cells were cultured in six-well plates in 2 ml of DMEM medium with 10% FBS and transfected with siRNAs at 40% confluence by lipofectamine 2000 (Invitrogen, Carls-bad, USA). The transfection was carried out twice in a row (one transfection per day). A final concentration of 50 nM siRNA duplexes was used in each transfection experiment.

Transfection, virion purification, western analyses, and subtilisin treatment
The 293T cells were transfected with viral DNAs using calcium phosphate [70]. Cells were lysed and supernatants were harvested 48 h post-transfection unless stated other-wise in the text. Centrifugation of virion particles and subtil-isin digestion of virions were performed as previously described [43]. Briefly, 293T cells were transfected with

10 µg of proviral DNA together with 2 µg of plasmid expressing HA-endophilin. The culture medium harvested from 293T cells 48 h post-transfection was filtered through a 0.45 µm filter and virions were purified on 25%/45% sucrose step gradient, followed by a second purification by sedimentation through a 25% sucrose cushion. For quantification of exogenously expressed endophilin and of the control protein HA-p11, in cytosolic and virion fractions, serial dilutions of cytosolic cell lysates and viral particles were prepared in SDS-PAGE sample buffer. Samples were loaded on the same SDS-acrylamide gel for electrophoresis and analyzed by western blotting with anti-HA antibody. To estimate the fraction of the cytosolic protein present in the virions, signal intensities in the lanes were compared and the dilution in the cytosolic series with the correspondingly closest signal to that seen in the virions was identified. The proportion in the virions was then calculated from the dilution giving comparable signals.

For subtilisin treatment, purified virion pellets were resuspended in subtilisin buffer (40 mM Tris pH 8, 2 mM $CaCl_2$) and incubated with various amounts of subtilisin (Boehringer Mannheim, Indianapolis, USA) for digestion overnight at room temperature. Reactions were stopped by adding PMSF and aprotinin to final concentrations of 2 mM and 1 µg/ml, respectively. Particles were then purified through a 25% sucrose cushion. For subtilisin digestion in the presence of detergent, NP-40 was added to a final concentration of 0.2%.

Exogenous reverse transcriptase assay
Exogenous reverse transcriptase assays were performed as described previously [43]. The amount of DNA synthesized was quantitated by PhosphorImager analysis of the radioactivity of the reverse-transcribed product.

Antibodies
Anti-mouse endophilin 2 polyclonal antibody was previously described [38]. We found it also cross-reacted with both the endogenous and the exogenously expressed human endophilin 2, but not with endophilin 1 (data not shown). Anti-dynamin 2 polyclonal antibody was from Santa Cruz Biotech (Santa Cruz, USA). Anti-HA monoclonal antibody was purchased from BabCO (Berkeley, USA). Anti-clathrin and AP-2 monoclonal antibodies were from Pharmingen (San Diego, USA). Goat polyclonal anti-CA antiserum (79S-804) was obtained from the National Cancer Institute (Bethesda, USA).

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