Cellular uptake of avian leukosis virus subgroup B is mediated by clathrin

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Abstract

Avian leukosis virus (ALV) requires endocytosis and a low pH step for successful viral entry. Here we report that transient treatment with lysosomotropic agents was not sufficient to block ALV subgroup B (ALV-B) entry, while it completely inhibited uptake of the pH-dependent Semliki Forest virus. Extended incubations with lysosomotropic agents were required to block ALV-B entry, suggesting that ALV particles are stable in endosomal compartments. We analyzed endocytic pathways involved in the uptake of ALV-B into target cells. The ALV-B receptor TVBS3 was not associated with detergent-resistant membranes (DRMs) in the presence or absence of ALV-B particles. This result suggested that DRM-associated endocytic pathways were not required for ALV-B entry. Using several approaches, we found that clathrin mediates endocytosis of ALV-B particles into target cells. By means of confocal microscopy, we established that the ALV-B receptor TVBS3 colocalized with clathrin in TVBS3-expressing quail QT-6 cells. In addition, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, blocked uptake of soluble ALV-B Env into chicken embryo fibroblasts. To examine ALV-B uptake into clathrin-negative cells, we used a chicken DT40 B cell line containing a tetracycline-regulatable clathrin gene. Clathrin depletion significantly reduced ALV-B entry into the chicken DT40 cell line. Taken together, our results suggest that clathrin is involved in uptake of ALV-B particles into target cells.

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Introduction

Infection of target cells by avian leukosis virus (ALV) has been used as a model system to study retroviral entry. The first step of ALV entry is binding of the viral envelope glycoprotein (Env) to a specific cellular receptor. ALV Env binding to its cognate receptor triggers a pH-independent conformational change of the viral Env (Earp et al., 2003; Matsuyama et al., 2004; Netter et al., 2004; White, 1995). Interestingly, lipid mixing of ALV and cellular membranes occurred at neutral pH (Earp et al., 2003). In addition, syncytia formation of ALV Env-expressing cells with ALV receptor-expressing cells was also described to occur at neutral pH (Earp et al., 2003). Several findings suggest that an additional pH-dependent step is required for productive ALV infection: (i) mild acidic conditions significantly enhance syncytia of viral Env-expressing cells with ALV receptor-expressing cells (Earp et al., 2003; Melikyan et al., 2004; Mothes et al., 2000); (ii) low pH in the presence of the cognate ALV receptor triggers the formation of stable ALV Env transmembrane protein oligomers (Mothes et al., 2000; Smith et al., 2004); and (iii) lysosomotropic agents, which prevent the acidification of endosomal compartments, inhibit ALV entry (Diaz-Griffero et al., 2002; Mothes et al., 2000). However, the endocytic machinery involved in ALV uptake remains to be identified.

ALV is divided into 10 major subgroups, designated A through J. Cellular receptors have been identified for ALV subgroups A, B, D, and E. The low-density lipoprotein (LDL)-like receptor TVA mediates entry of ALV-A (Bates et
al., 1993), and a family of TNFR-related receptors (TVBS\textsuperscript{S3}, TVBS\textsuperscript{L1}, and TVB\textsuperscript{L}) confers susceptibility to ALV subgroups B, D, and E (Adkins et al., 1997, 2000; Brojatsch et al., 1996). TVBS\textsuperscript{S3}, the receptor for cytopathic ALV-B/D, contains 3 extracellular cysteine-rich domains, a transmembrane-spanning domain, and a cytoplasmic region, which includes an 80 amino acid “death domain” that is essential for ALV-B-mediated cytopathic effects (Diaz-Griffero et al., submitted for publication; Tartaglia et al., 1993). The ALV-B Env binds to a linear extracellular epitope of TVBS\textsuperscript{S3} corresponding to residues 32–46 (Knauss and Young, 2002). The native ALV Env is a trimer of heterodimers, which are composed of the surface unit (SU) and the transmembrane unit (TM). ALV Env TM presents structural similarities with the Env of influenza A virus, specifically the hemagglutinin 2 subunit (HA-2). The influenza A virus is a highly characterized pH-dependent virus, and low pH triggers a conformational change of the HA-2 protein (Skehel and Wiley, 2000; Weissenhorn et al., 1999). This conformational change triggers the exposure of a fusion peptide, which mediates virus-to-cell membrane fusion (Bullough et al., 1994; Carr and Kim, 1993; Durrer et al., 1996; Stegmann et al., 1990).

The low pH environment of endocytic compartments is required for successful fusion by certain viruses (Maxfield and Yamashiro, 1987; McClure et al., 1990). The pH-dependence suggests that endocytosis of viral particles to acidic compartments is required for productive infection. Endocytosis appears to be essential for productive ALV infection, as dominant-negative dynamin blocks ALV entry (Mothes et al., 2000), and ALV particles are found in endosomes (Diaz-Griffero et al., 2002).

Several types of endocytic pathways have been described for viral uptake into target cells, including clathrin-mediated endocytosis, detergent-resistant membrane (DRM)-mediated endocytosis, macropinocytosis, and phagocytosis (Sieczkarski and Whittaker, 2002; Smith et al., 2004). Clathrin-mediated endocytic pathways are involved in the uptake of several enveloped viruses, including Sindbis virus (DeTullio and Kirchhausen, 1998), Semliki Forest virus (SFV) (Doxsey et al., 1997), and human polyoma virus JC virus (Pho et al., 2000; Querbes et al., 2004). Interaction of clathrin-dependent viruses with surface receptors triggers the sequential recruitment of downstream adaptor proteins and the formation of clathrin-coated pits. Subsequently, clathrin-coated vesicles transfer viral particles to the acidic environment of endosomes, where viral fusion occurs (Marsh and Pelchen-Matthews, 2000). Some viruses, including simian virus 40, polyoma virus, and ALV-A (Narayan et al., 2003; Pelkmans and Helenius, 2002), can be endocytosed into target cells via DRMs, such as caveolae or rafts. The GPI-anchored form of the ALV-A receptor, TVA800, is associated with DRMs (Bates et al., 1993; Narayan et al., 2003). Caveolae are characterized by their enrichment in sphingolipids, cholesterol, and cholesterol binding proteins (Kurzchalia and Parton, 1999). Clathrin-independent and caveolae-independent endocytic pathways are involved in the uptake of influenza virus and SV40 (Damm et al., 2005; Sieczkarski and Whittaker, 2002). Other viruses, including adenovirus and HIV, can be internalized via macropinocytosis, an endocytic mechanism mediated by the formation of large vesicles (Marechal et al., 2001; Meier et al., 2002).

In this study, we demonstrated that extended treatment of target cells with lysosomotropic agents is required to inhibit ALV-B entry while transient exposure to these agents proves insufficient. These results suggest that ALV-B particles are stable in endosomal compartments in the presence of lysosomotropic agents. We found that ALV-B uses clathrin-mediated endocytic pathways to enter target cells. This was supported by experiments showing that clathrin colocalized with the ALV-B receptor TVBS\textsuperscript{S3} and that clathrin depletion blocked ALV-B entry into avian cell lines. In contrast to clathrin, DRM-associated caveolin-1 was not required for TVBS\textsuperscript{S3} trafficking and ALV-B entry.

**Results**

**ALV-B is stable in intracellular compartments**

Use of lysosomotropic agents, which prevent the acidification of endosomal compartments, inhibit productive ALV infections (Diaz-Griffero et al., 2002; Earp et al., 2003; Mothes et al., 2000). The efficiency of lysosomotropic agents in blocking ALV entry is subgroup-specific and receptor-dependent. Transient treatment with lysosomotropic agents was only minimally effective in blocking ALV-A entry into cells expressing the ALV-A receptor TVA800 (Narayan et al., 2003). We hypothesized that extended incubation times with lysosomotropic agents are required for productive ALV infection. To test this, we challenged human HEK 293 cells expressing the ALV-B receptor TVBS\textsuperscript{S3} with pseudotyped murine leukemia virus (MLV) particles bearing the envelope glycoprotein (Env) of ALV-B. As controls, we used MLV particles bearing either the Env of pH-dependent SFV or pH-independent amphotropic MLV. The pseudotyped MLV particles express the firefly luciferase protein upon infection. 293-TVB\textsuperscript{S3} cells were infected with pseudotyped MLV particles in the presence of the lysosomotropic agent NH\textsubscript{4}Cl. At different time points, the NH\textsubscript{4}Cl-containing media were replaced with drug-free media, and luciferase activity was measured 48 h postinfection. As expected, entry of MLV particles pseudotyped with SFV Env into 293-TVB\textsuperscript{S3} cells was blocked efficiently in the presence of NH\textsubscript{4}Cl. Incubation times of 1 h with lysosomotropic agents were sufficient to completely block (>95%) entry of SFV pseudotypes, indicating rapid and efficient inhibition of SFV entry by these agents (Fig. 1A).

In contrast to SFV pseudotypes, entry of MLV particles pseudotyped with ALV-B Env into 293-TVB\textsuperscript{S3} cells
required significantly longer incubation times with NH$_4$Cl.
Ammonium chloride treatment for 2 h or less was insufficient to block entry of ALV-B Env pseudotyped viruses. Approximately 8 h of NH$_4$Cl treatment was required to inhibit more than 80% of ALV-B entry (Fig. 1B). In agreement, we have previously reported that ALV-B entry was efficiently blocked after 4 h of treatment with NH$_4$Cl (Diaz-Griffero et al., 2002).

Prolonged ammonium chloride treatment was not toxic, as NH$_4$Cl treatment of 293-TVBS$_3$ cells for up to 24 h did not inhibit infection of MLV particles pseudotyped with the amphotropic MLV Env (Fig. 1C). Intriguingly, NH$_4$Cl treatment increased infection of amphotropic MLV Env-pseudotyped viruses.

These results indicated that ALV-B particles are stable in endosomal compartments in the presence of lysosomotropic agents and remain infectious after transient treatment with NH$_4$Cl. To understand the mechanism by which ALV-B particles are internalized, we investigated endocytic pathways involved in ALV-B entry.

**DRM-associated proteins are not involved in TVBS$_3$-trafficking**

The ALV-B receptor TVBS$_3$ is a member of the TNFR family. The mammalian TNFR-1 is associated with DRMs through the cytoplasmic death domain (Cottin et al., 2002; Jones et al., 1999). To test whether TVBS$_3$ is associated with DRMs, we performed flotation assays. DF-1 cells were lysed with a Triton X-100 containing buffer and cellular lysates were subjected to a discontinuous sucrose density gradient centrifugation (5–40%). DRM-associated proteins, such as caveolin-1, float towards the top of the sucrose gradient, whereas detergent-soluble fractions move to the bottom of the gradient (Scherer et al., 1994). Following ultracentrifugation, protein fractions were collected and analyzed for TVBS$_3$ and caveolin-1 expression by Western blotting using SUB-IgG and anti-caveolin-1 antibodies, respectively. TVBS$_3$ was found predominantly in the detergent-soluble fractions 8 through 12, while caveolin-1 was present in detergent-insoluble fractions 3 through 9 (Fig. 2A). The differential fractionation of TVBS$_3$ and caveolin-1 indicated that TVBS$_3$ was not associated with DRMs (Fig. 2A). In the case of the glial cell-derived neurotropic receptor and the MHC class II receptor, ligand binding triggers translocation of these receptors to DRMs (Simons and Toomre, 2000). Therefore, we tested whether binding of ALV-B particles alters the cellular localization of TVBS$_3$ and recruitment to DRMs. We found that TVBS$_3$ did not shift towards the insoluble fractions in the presence of high titer ALV-B particles (Fig. 2B). Our findings are consistent with the susceptibility to ALV-B infection of caveolin-1-negative human astrocytoma (U-251) cells expressing the ALV-B receptor TVBS$_3$ (Diaz-Griffero et al., 2002). These results suggested that TVBS$_3$ is not associated with DRMs and that ALV-B entry does not require DRMs. This is in contrast to ALV-A, which could enter target cells via the DRM-associated GPI-anchored ALV-A receptor TVA800 (Narayan et al., 2003).

**TVBS$_3$ colocalized with clathrin**

We have previously observed ALV-B particles in clathrin-coated vesicles, suggesting involvement of clathrin in ALV-B entry (Diaz-Griffero et al., 2002). In order to test the role of clathrin in TVBS$_3$ trafficking and ALV-B entry, we investigated whether TVBS$_3$ is associated with clathrin.
For this purpose, we cloned the TVB$^{S3}$ full-length cDNA into the mammalian expression vector pEF6/V5-His TOPO. This construct fused a 14-amino acid V5 epitope and a polyhistidine (6xHis) tag to the C-terminus of TVB$^{S3}$. The V5-tagged TVB$^{S3}$-fusion protein was stably expressed in QT-6 cells (Figs. 3A and B) and used for colocalization studies. TVB$^{S3}$ and clathrin heavy chains were visualized by using anti-V5 and anti-clathrin antibodies, respectively. Confocal images illustrated extensive colocalization of TVB$^{S3}$ with the clathrin heavy chain in TVB$^{S3}$-expressing QT-6 cells (Figs. 3A and B). TVB$^{S3}$ and clathrin shared punctuate staining on the plasma membrane of these cells (Fig. 3A, see inserted box). Cross-sections of TVB$^{S3}$-expressing QT-6 cells revealed perinuclear localization of the receptor with clathrin (Fig. 3B).

Intracellular clathrin has been reported to localize to the trans-Golgi network (TGN) (Carreno et al., 2004). Moreover, other members of the death receptor family such as TNFR-1 are predominantly associated with the TGN (Jones et al., 1999). Therefore, we tested whether intracellular TVB$^{S3}$ is associated with the TGN. Since TGN markers are better characterized in human cells, we performed localization experiments in our previously described human U-251 cells expressing TVB$^{S3}$ (Diaz-Griffero et al., 2002). We found that perinuclear TVB$^{S3}$ colocalized extensively with the TGN marker TGN46 (Fig. 3C). TGN localization of TVB$^{S3}$ was confirmed by using brefeldin A, an inhibitor of anterograde vesicular transport from the ER to the TGN and between TGN stacks (Lippincott-Schwartz et al., 1990). Brefeldin A treatment collapses the majority of the TGN around the microtubule organizing center (Reaves and Banting, 1992). Brefeldin A (5 μg/ml) treatment of TVB$^{S3}$-expressing astrocytoma cells for 1 h resulted in perinuclear accumulation of TVB$^{S3}$ and colocalization with the TGN (Fig. 3D). Taken together, our results indicated that TVB$^{S3}$ colocalizes with clathrin on the plasma membrane and with the TGN in intracellular compartments.

**Clathrin inhibitors block internalization of soluble ALV-B Env**

We have previously shown that chlorpromazine, an inhibitor of clathrin-mediated endocytosis, blocks ALV-B infection (Diaz-Griffero et al., 2002). This cationic amphiphilic drug disrupts endocytosis by relocating clathrin and the adaptor protein AP2 from the cell surface (Wang et al., 1993). To determine whether chlorpromazine blocked ALV entry by inhibiting endocytosis of TVB$^{S3}$, we performed internalization studies using a soluble form of the ALV-B Env protein (SUB-IgG) by confocal microscopy. SUB-IgG
is composed of the SU part of ALV-B Env cloned in frame to the Fc portion of the rabbit IgG heavy chain (Brojatsch et al., 1996). After prebinding DF-1 cells with purified SUB-IgG at 4 °C, endocytosis of soluble ALV-B Env was induced by shifting the temperature to 37 °C. ALV-B Env internalization in the presence or absence of chlorpromazine was analyzed by confocal microscopy using Cy3-labeled secondary anti-rabbit antibodies.

After incubation at 4 °C, SUB-IgG was present predominantly at the plasma membrane (Fig. 4A), and upon shifting the temperature to 37 °C, we observed perinuclear localization of SUB-IgG, indicating internalization of ALV-B Env (Figs. 4B and C). In contrast, internalization of SUB-IgG was not detected in chlorpromazine-treated cells (Figs. 4D–F). This result is consistent with our previous findings that chlorpromazine efficiently inhibited ALV-B entry into target cells (Diaz-Griffero et al., 2002; Mothes et al., 2000). Chlorpromazine blockage of ALV-B Env uptake suggested that this process is mediated by clathrin.

**Clathrin depletion inhibits ALV-B uptake**

As a final and more conclusive approach to study clathrin involvement in ALV-B entry, we employed a genetic strategy. To deplete cells of the clathrin heavy chain protein, we took advantage of a chicken B cell line, DT40, which was engineered to lack both endogenous alleles of clathrin. These clathrin-negative cells express the human clathrin heavy chain under the control of a tetracycline-regulatable promoter (Tet-Off system) (Wettey et al., 2002). The tetracycline-regulatable promoter controls the firefly luciferase gene, in addition to the clathrin heavy gene, to monitor the efficiency of these regulatory regions. Doxycycline treatment completely abrogated luciferase expression in two independent DT40 cell lines, DKO-S and DKO-R cells (Fig. 5A). These DT40 clones differ in their responsiveness to clathrin depletion. Extended clathrin depletion kills DKO-S cells, while DKO-R cells remain viable in the absence of clathrin for several days (Wettey et al., 2002). To address the role of clathrin in ALV-B entry, we depleted DKO-S and DKO-R cells of clathrin by treatment with 1 μg/ml of doxycycline. Doxycycline treatment suppressed clathrin expression in both cell lines 96 h after treatment (Fig. 5B). Clathrin-depleted cells were challenged by ALV-B expressing the green fluorescent protein (ALV-B-eGFP) at a multiplicity of infection of 5. In the case of DKO-S cells, we removed doxycycline 24 h after infection to ensure survival of these cells. ALV-B-eGFP uptake was measured by analyzing GFP expression using flow cytometry 48 h postinfection. In both cell lines, clathrin depletion led to a significant reduction of ALV-B uptake (Fig. 5C). As expected, clathrin depletion did not affect entry of amphotropic MLV (Fig. 5C), suggesting that the inhibition of ALV-B entry is specific and not due to pleiotropic effects caused by clathrin depletion.

Residual ALV-B uptake into clathrin-depleted DT40 cells was presumably mediated by alternative pathways, including clathrin-independent pinocytosis, which are upregulated 30 min after clathrin-mediated endocytosis is inhibited (Damke et al., 1995). Consistent with our findings, recycling of chicken transferrin, which is mediated by clathrin, is reduced to a similar extent after clathrin elimination, suggesting residual trafficking of transferrin via clathrin-independent pathways (Wettey et al., 2002). Taken together,

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**Fig. 4. Uptake of soluble ALV-B Env is blocked by chlorpromazine.** DF-1 cells were incubated with 50 ng of soluble ALV-B Env (SUB-IgG) for 1 h at 4 °C. Internalization was measured in the presence (A–C) or absence (D–F) of chlorpromazine 50 μM 0, 30, and 60 min postinfection. Soluble ALV-B Env was immunostained using an anti-rabbit Cy3-labeled antibody as described in Materials and methods. The cellular nucleus is indicated by “n”.

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our results suggested that both a low pH step and clathrin-mediated endocytosis are crucial for ALV-B entry into target cells.

Discussion

Entry of pH-dependent viruses into target cells is a multistep process where cell attachment and internalization represent spatio-temporally distinct steps. Mounting evidence suggests that ALV entry into target cells is a two-step process. Binding of ALV Env to its cognate receptor triggers a pH-independent conformational change, which is followed by endocytosis of ALV particles (Diaz-Griffero et al., 2002; Mothes et al., 2000). Previous reports have shown that lipid mixing, an early stage of viral fusion, is non-responsive to lysosomotropic agents (Earp et al., 2003; Gilbert et al., 1990). However, productive ALV infection requires a low pH step (Diaz-Griffero et al., 2002; Mothes et al., 2000; Narayan et al., 2003), and it remains to be shown whether a low pH phase promotes a step prior to lipid mixing or is required for pore formation (Earp et al., 2003). We found that transient treatment with lysosomotropic agents was insufficient to block ALV-B entry, and treatment for at least 8 h was required to inhibit ALV-B uptake significantly. This in agreement with a previous report showing that transient treatment with lysosomotropic agents does not block ALV-A entry (Earp et al., 2003), while continuous treatment with these agents strongly inhibits ALV-A and ALV-B entry (Barnard et al., 2004; Mothes et al., 2000; Narayan et al., 2003). However, lysosomotropic agents do not block lipid mixing of ALV membranes and target cell membranes (Earp et al., 2003; Gilbert et al., 1990). This suggests that ALV-B particles are stable in intracellular compartments and that early replacement of lysosomotropic drugs allows fusion of viral particles in intracellular compartments. Our findings show that ALV-B is stable in endocytic compartments, consistent with experiments described for ALV-A (Earp et al., 2003; Narayan et al., 2003). Presumably, lysosomotropic agents prevent ALV-B fusion with endocytic membranes and arrest ALV-B particles in an infectious state. The slow kinetics, by which lysosomotropic agents block ALV-B entry, are in stark contrast to the effect of these drugs on SFV uptake. SFV entry is completely abrogated by short treatment with these inhibitors, suggesting that SFV, unlike ALV-B, is endocytosed to a different intracellular compartment, where it is rapidly inactivated, even in the presence of lysosomotropic agents (Diaz-Griffero et al., 2002). The stability of ALV-B in endosomes is shared by ALV-A, when ALV-A is internalized by TVA800 (Narayan et al., 2003). ALV-A entry is mediated by two alternative receptors, TVA800 and TVA950. ALV-A particles internalized via the GPI-anchored TVA800 are stable in intracellular compartments and remain highly infectious for more than 6 h in the presence of the lysosomotropic agent ammonium chloride (Narayan et al., 2003). In contrast, ALV-A, endocytosed via TVA950, is rapidly degraded in the presence of ammonium chloride (Narayan et al., 2003). Taken together, our results support the hypothesis that ALV entry is pH-dependent and that extended incubations with lysosomotropic agents are required to block ALV entry. It is conceivable that ALV particles undergo hemifusion at neutral pH (Earp et al., 2003) and that low pH is required to open fusion pores and complete the fusion process.

Lysosomotropic agents arrest ALV-B particles in endosomes and appear to prevent lysosomal degradation of these viral particles. Endocytosis and lysosomal degradation also play a role in entry of MLV and HIV. For example, the
infectivity of HIV-1 is increased in the presence of lysosomotropic agents, suggesting that HIV is rapidly endocytosed and degraded in lysosomes (Frederickson et al., 2002). In agreement with this, we demonstrated that the infectivity of amphotropic MLV increases upon treatment with lysosomotropic agents (Fig. 1C). Aside from degradation of endocytosed viruses in lysosomal compartments, it has been suggested that infectious viral particles can also be stored in intracellular compartments (Geijtenbeek et al., 2000). For example, HIV-1 particles accumulate in MHC class II compartments of macrophages (Raposo et al., 2002) and can be stored in intracellular compartments of dendritic cells for several days to infect T cells subsequently (Kwon et al., 2002; McDonald et al., 2003).

The pH dependence of ALV fusion suggests that endocytosis provides the acidic environment for ALV fusion and productive infections. Several findings suggest that endocytosis of ALV-B particles is essential for productive viral infections: (i) ALV entry is pH-dependent, as shown by inhibition studies using lysosomotropic agents and by synctia formation of ALV-infected cells at acidic pH (Diaz-Griffero et al., 2002; Melikyan et al., 2004; Mothes et al., 2000); (ii) dominant-negative dynamin reduces ALV entry into target cells (Mothes et al., 2000); (iii) ALV particles are found in endocytic compartments (Diaz-Griffero et al., 2002). Despite promoting ALV fusion, we have preliminary evidence that pH could play an additional role in ALV entry. We found that protease inhibitors block ALV-B entry (data not shown), suggesting that proteases are involved in this process. The proteolytic activity of several cellular proteases is pH dependent and could be blocked by lysosomotropic agents. We are currently investigating the role of cellular proteases in ALV-B entry.

Several lines of evidence suggest that ALV-B uptake is clathrin-dependent: (i) the ALV-B receptor TBV3 colocalized with clathrin; (ii) ALV particles were found in clathrin-coated vesicles in chicken embryo fibroblasts (Diaz-Griffero et al., 2002); (iii) uptake of ALV-B particles (Diaz-Griffero et al., 2002) and soluble ALV-B Env into target cells was blocked by the clathrin-mediated uptake inhibitor chlorpromazine; and (iv) transient clathrin depletion reduced ALV-B entry into target cells.

Materials and methods

Cell lines, reagents, and antibodies

DF-1 chicken embryo fibroblasts, human 293T cells expressing TBV3, and human U-251 astrocytoma cells expressing TBV3 have been described previously (Diaz-Griffero et al., 2002). Cells were grown in complete Dulbecco’s modified Eagle media (DMEM media; Mediatech) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Ammonium chloride, chlorpromazine, brefeldin A, and doxycycline were obtained from Sigma. Fifty millimolar ammonium chloride, 50 µM chlorpromazine, 5 µg/ml brefeldin A, and doxycycline (1 µg/ml) were used in the inhibition experiments. These inhibitors did not affect the pH of the cellular media at the indicated concentrations.
Mouse monoclonal serum anti-clathrin heavy chain clone TD.1 isotype IgG2a was obtained from Sigma-Aldrich. Mouse monoclonal serum anti-V5 isotype IgG1 was obtained from Invitrogen. Monoclonal serum anti-mouse IgG1-alexa 555 and IgG2a-alexa 488 were obtained from Molecular Probes. Monoclonal serum against TGN 46 was obtained from Zymed. Serum anti-rabbit Cy2-labeled and anti-mouse Cy2-labeled antibodies were obtained from Jackson Immunoresearch. Two DT40 cell lines, DKO-S and DKO-R cells, lack both endogenous clathrin alleles and express the human clathrin heavy chain under the control of a tetracycline-regulatable promoter (Tet-Off system) (Wetey et al., 2002). DKO-S cells are highly sensitive to clathrin depletion, while DKO-R cells remain viable for several days in the absence of clathrin (Wetey et al., 2002). These cell lines were used as described previously (Wetey et al., 2002). ALV-B virus was produced by transfecting DF-1 cells with pRCASBP(B)-eGFP (Mothes et al., 2000). 2002). ALV-B virus was produced by transfecting DF-1 cells with pRCASBP(B)-eGFP (Mothes et al., 2000). Supernatants were collected 48 h after transfection and stored at −80 °C. Viral titers were determined by endpoint dilution assays.

Production of MLV pseudotyped particles

A total of $4 \times 10^6$ GP-2-293 cells seeded on 100-mm tissue culture plates were cotransfected with 5 μg of pLAPSN (Luciferase reporter; Clontech) or 5 μg of QNCeGFP (eGFP reporter; Clontech) and 5 μg of envelope-expressing plasmids using the calcium phosphate procedure as described in the manual for the pantropic Retroviral Expression System (Clontech). GP-2-293 cells stably express gag and pol genes of murine leukemia virus (Clontech). Pseudotyped particles were collected 48 h after transfection and stored at −80 °C. SFV Env and murine leukemia virus (amphotropic MLV) Env-expressing plasmids were obtained from Tanya Dragic (Albert Einstein College of Medicine, Bronx, NY). Pseudotype viruses expressing the eGFP as a reporter were tittered by endpoint dilution assays.

Viral entry and luciferase assays

Pseudotyped viruses were prebound to $1 \times 10^5$ cells on 24-well tissue culture plates at 4 °C for 1 h. Supernatants were replaced with fresh media containing lysosomotropic agents and incubated at 37 °C for different times. Subsequently, media were replaced with complete medium, and cellular extracts were prepared 48 h after infection to assay for luciferase activity. Extracts were made using 250 μl of luciferase lysis buffer (Promega), and after 1 freeze/thaw cycle, samples were centrifuged at 12,000 × g for 5 min. Ninety microliters of the supernatant was mixed with 30 μl of the luciferase substrate (Promega) and incubated for 3 min at room temperature. Luciferase activity was measured using a luminometer (Perkin-Elmer Wallac). To assay entry of ALV-B in DKO-R and DKO-S cells, we used flow cytometry. A total of $1 \times 10^5$ infected DKO-R or DKO-S cells were analyzed with a FACSCalibur flow cytometer in the FL-1 channel to detect infected cells (GFP-positive).

Colocalization experiments

For colocalization studies, we cloned the TVB3 full-length cDNA into the mammalian expression vector pEF6/V5-His TOPO (Invitrogen). TVB3 full-length cDNA was amplified using PCR with the following oligonucleotide primers: FD11 (TVB35′) 5′GGACCAAAACCGTTCC-TAACTCGG; FD12 (TVB33′) 5′GCTCACCTCG-TACTGGAAATAGCCC. The PCR-amplified fragment was cloned into pEF6/V5-His TOPO following manufacturer directions (Invitrogen). This construct was transfected into QT-6 cells using lipofectamine (Invitrogen). QT-6 cells stably expressing the tagged receptor were selected by using Blastidcin 3 μg/ml (Invitrogen). Single-cell clones were obtained by seeding 30 cells on 96-well plates. Cells were grown overnight on 12-mm diameter coverslips and fixed in 3.9% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Cellgro) for 30 min. Cells were washed in PBS, incubated in 0.1 M glycine (Sigma) for 10 min, washed in PBS, and permeabilized with 0.05% saponin (Sigma) for 30 min. Samples were blocked with 10% donkey serum (Dako) for 30 min and incubated for 1 h with primary antibodies. Subsequently, cells were washed with PBS and incubated with secondary antibodies. Samples were mounted for fluorescence microscopy by using the ProLong Antifade Kit (Molecular Probes). Images were acquired by using a BioRad Radiance 2000 laser scanning confocal microscope with Nikon 60 × N.A.1.4 optics.

Immunoblotting

Cell lines were grown on 100-mm plates to a confluency of 90%, and cell extracts were prepared with a homogenization buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 1 mM EDTA) containing a cocktail of protease inhibitors (Roche, Indianapolis, ID). Cells were subjected to Dounce-homogenization and centrifuged at 2,500 × g to obtain postnuclear supernatants. We determined protein concentration using a bicinchoninic acid protein assay reagent kit (Pierce). Ten micrograms of protein was applied to a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with SUB-IgG (to detect TVB3), anti-clathrin heavy chain antibodies, and anti-caveolin-1 antibodies. Subsequently, filters were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulins as secondary antibodies (Amersham Pharmacia). Antibody binding was detected by using an enhanced chemiluminescence kit (Amersham Pharmacia).
**Fractionation of detergent-resistant membranes**

Cells were grown on 12-mm coverslips and incubated with SUB-IgG at 4 °C for 1 h. Internalization was induced by incubating cells at 37 °C in the presence or absence of 50 μM chlorpromazine for different times. Samples were fixed in 3.9% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 30 min. Cells were washed with PBS containing 0.1 M glycine (Sigma) for 10 min, washed with PBS, and permeabilized with 0.05% saponin (Sigma) for 30 min. Samples were blocked with 10% donkey serum for 30 min (Dako), and SUB-IgG was identified using anti-rabbit Cy3-labeled secondary antibodies (Jackson Immunoresearch). Samples were mounted for fluorescence microscopy on coverslips by using the ProLong Antifade Kit (Molecular Probes). Images were acquired by using a BioRad Radiance 2000 laser scanning confocal microscope with Nikon 60 × N.A. 1.4 optics.

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