Cell, Vol. 103, 679–689, November 10, 2000, Copyright ©2000 by Cell Press

Retroviral Entry Mediated by Receptor Priming and Low pH Triggering of an Envelope Glycoprotein

Walther Mothes,*† Adrienne L. Boerger,* Shakti Narayan,‡ James M. Cunningham,*† and John A. T. Young*‡§

*Department of Microbiology and Molecular Genetics
Harvard Medical School
Boston, Massachusetts 02115

†Howard Hughes Medical Institute and Division of Hematology and Oncology
Brigham and Women's Hospital and Harvard Medical School
Boston, Massachusetts 02115

‡McArdle Laboratory for Cancer Research
University of Wisconsin-Madison
Madison, Wisconsin 53706

Summary

Avian leukosis virus (ALV) has been used as a model system to understand the mechanism of pH-independent viral entry involving receptor-induced conformational changes in the viral envelope (Env) glycoprotein that lead to membrane fusion. Here, we report the unexpected finding that ALV entry depends on a critical low pH step that was overlooked when this virus was directly compared to the classical pH-dependent influenza A virus. In contrast to influenza A virus, receptor interaction plays an essential role in priming ALV Env for subsequent low pH triggering. Our results reveal a novel principle in viral entry, namely that receptor interaction can convert a pH-insensitive viral glycoprotein to a form that is responsive to low pH.

Introduction

Entry of enveloped viruses into cells is mediated by viral fusion proteins. These proteins, exemplified by the influenza A virus hemagglutinin (HA) and retroviral envelope (Env) proteins, consist of trimers of heterodimers, HA_1 and HA_2, as well as surface (SU) and transmembrane (TM) subunits, respectively (for review, see Hernandez et al., 1996). The HA_1 and SU proteins mediate cell attachment through receptor binding and HA_2 and TM proteins mediate fusion of viral and cellular membranes. Moreover, structural studies have demonstrated a striking similarity between retroviral TM proteins and HA_2, suggesting a conserved fusion mechanism (reviewed in Weissenhorn et al., 1999).

Despite these similarities, the entry mechanisms of influenza A virus and of most retroviruses differ in their requirement for a low pH environment. Following binding of influenza A virus HA_1 to cell surface sialic acid molecules, influenza viruses are taken up by endocytosis and trafficked to a low pH endosomal compartment where HA_2 mediates membrane fusion. Low pH triggers a series of conformational changes that lead to the exposure and insertion of the fusion peptide into the cellular membrane (Stegmann et al., 1990; Carr and Kim, 1993; Bullough et al., 1994; Durrer et al., 1996). Viral and cellular membranes are likely brought together for fusion by the formation of a highly stable hairpin-like structure in which the N-terminal fusion peptide and the membrane-spanning domain are located at the same end of the HA_2 molecule (Weissenhorn et al., 1999).

Most retroviruses are thought to use a pH-independent entry mechanism (Hernandez et al., 1996; Sodroski, 1999). One of the best studied examples is the avian leukosis virus (ALV). Its entry mechanism was classified as pH-independent in comparison to influenza A virus. In contrast to influenza A virus, which was rapidly inactivated at low pH due to premature activation of HA (Puri et al., 1990), a subgroup C ALV was not affected by this treatment (Gilbert et al., 1990). Whereas influenza A virus induced cell–cell fusion after a brief exposure to low pH, subgroup C ALV did not (Gilbert et al., 1990). Furthermore, transient treatment of cells with lysosomotropic agents that neutralized acidic endosomal compartments inhibited influenza A virus infection but did not appear to affect infection by subgroups A and C ALV (Gilbert et al., 1990).

Consequently, it has been proposed that receptor binding suffices to induce conformational changes in the ALV Env that drive fusion at the plasma membrane. Consistent with that model, a soluble form of Tva, the cellular receptor for subgroup A-ALV (ALV-A) (Bates et al., 1993), induced temperature-dependent, conformational changes in ALV-A Env (EnvA), resulting in an increased protease-sensitivity of SU, exposure of the fusion-peptide regions of TM, and stable binding of TM to liposomes (Gilbert et al., 1995; Hernandez et al., 1997; Damico et al., 1998).

Here we demonstrate, that in contrast to these findings, ALV entry does require a low pH step that acts downstream of receptor binding. This work defines novel criteria for characterizing the pH-dependence of viral entry and demonstrates a novel principle in enveloped viral entry, namely that receptor can prime the viral fusion protein for low pH-activated fusion.

Results

A PCR-Based Assay to Monitor ALV Reverse Transcription Following Cell Entry

A standard PCR-based assay (Zack et al., 1990) was used to monitor reverse transcription of the ALV genome immediately following viral entry into cells. Two sets of ALV-specific oligonucleotide primers were generated (derived from the long terminal repeat [LTR] region and from the polymerase [*pol*] region) that allowed for detection of intermediate viral DNA products (Figure 1A). To follow the kinetics of ALV DNA synthesis during an infection, a subgroup A and a subgroup B ALV (ALV-A and ALV-B) were bound on ice to chicken DF-1 cells that express ALV receptors and also to mammalian 293 cells

§To whom correspondence should be addressed (e-mail: young@oncology.wisc.edu).

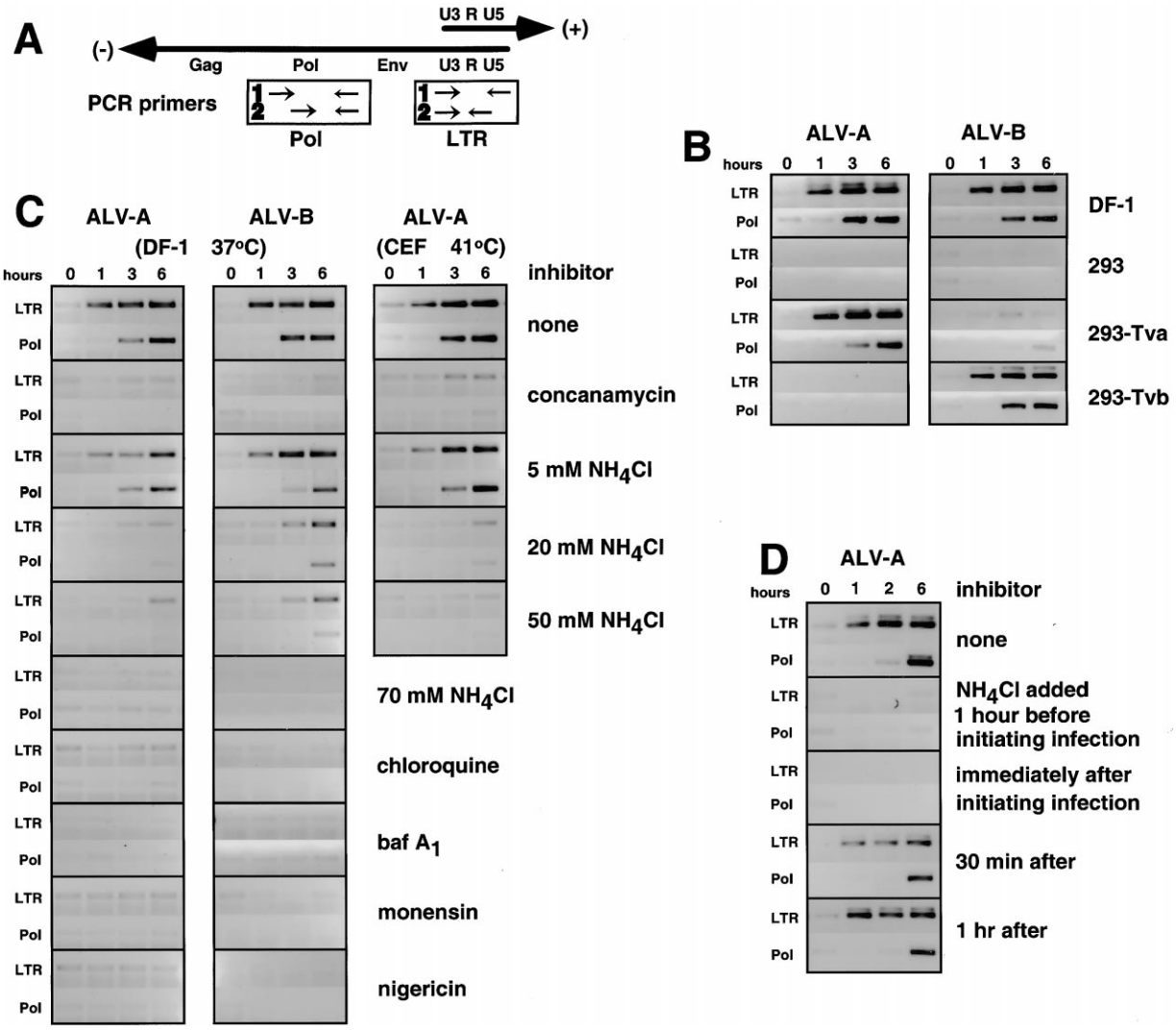


Figure 1. Lysosomotropic Agents Block an Early Step of ALV Entry

(A) A DNA intermediate of retroviral reverse transcription (Telesnitsky and Goff, 1997) is shown along with oligonucleotide primers specific for the LTR and *pol* regions that were used for semi-nested PCR amplification of viral DNA products (B–E).

(B) Chicken DF-1 cells and human 293 cells that did or did not express viral receptors Tva and Tvb were incubated on ice with ALV-A and ALV-B and infection was then initiated by incubation at 37°C. Aliquots of the cells were taken and the DNA prepared immediately before the temperature shift (0 hr), and at 1, 3, or 6 hr after incubation at 37°C. Semiquantitative PCR was performed using the LTR- and *pol*-specific primers. The PCR-amplified products were visualized on an ethidium bromide–stained agarose gel using a BioRad FluorS imager.

(C) Chicken DF-1 cells and primary chicken embryonic fibroblasts (CEF) were infected by ALV (at the indicated temperature and as described in the legend to Figure 1B), in the absence of inhibitors (none) or in the constant presence of 20nM concanamycin, increasing amounts of ammonium choride (5–70 mM NH_4Cl), 0.3 mM chloroquine, 200 nM bafilomycin (baf A_1), 30 μM monensin and 5 μM nigericin.

(D) The upper two panels show a standard infection in the absence or presence of 70 mM ammonium chloride (added one hour before initiating virus infection). In the lower three panels, 70 mM ammonium chloride was added to cells immediately after, 30 min after, or one hour after initiating infection.

that did or did not express the ALV receptors, Tva and Tvb (Bates et al., 1993; Brojatsch et al., 1996; Adkins et al., 2000). After removal of unbound virus, aliquots of these cells were harvested for viral DNA analysis either immediately (0 hour samples; Figure 1B), or after shifting the temperature to 37°C for 1, 3, or 6 hr (Figure 1B) to allow infection. Consistent with the temporal progression of reverse transcription, ALV LTR-specific DNA products were detected earlier than the corresponding *pol*-specific DNA products (Figure 1B). The appearance of viral DNA required cognate ALV Env-receptor interactions as demonstrated with transfected 293 cells (Figure 1B). These data show that the PCR-based assay reliably monitors reverse transcription following receptor-dependent ALV entry.

Lysosomotropic Agents Block an Early Step in ALV Replication

Using the PCR entry assay, we were surprised to find that the constant presence of lysosomotropic agents blocked the appearance of viral DNA in DF-1 cells and primary chicken embryonic fibroblasts (CEFs) chal-

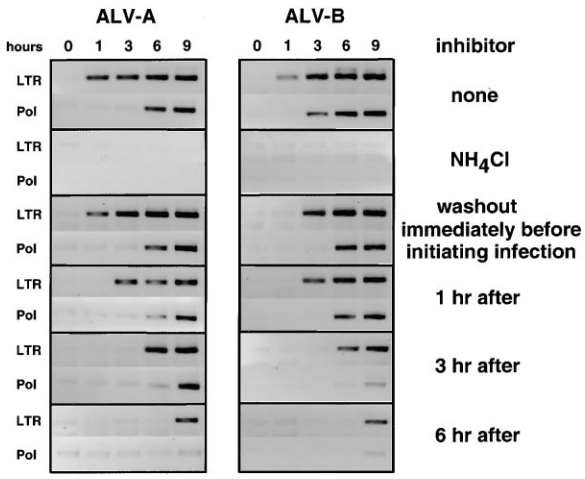


Figure 2. ALV Is Stable in Ammonium Chloride–Treated Cells

Virus/cell complexes were generated on ice in the absence (none, upper panel) or presence (lower panels) of 70 mM ammonium chloride (NH_4Cl). Ammonium chloride was washed out immediately before the infection was initiated at 37°C (0 hr), or at 1, 3, and 6 hr later. DNA samples were analyzed as in Figure 1B.

lenged with ALV viruses (Figure 1C). The agents used include the weak bases, ammonium chloride and chloroquine; inhibitors of the vacuolar H^+/ATPase, concanamycin and bafilomycin A_1; and the ionophores, monensin and nigericin. Control PCR experiments performed using oligonucleotide primers specific for the chicken β-actin gene confirmed that DNA was present in all samples (data not shown). This block was observed when the inhibitor was added either one hour before, or immediately after, initiating ALV-A infection, but not 30 or 60 min later (Figure 1D for ammonium chloride). Since the common property of these different lysosomotropic agents is to elevate the pH within endosomal compartments (Duve et al., 1974; Drose and Altendorf, 1997), these data suggest unexpected roles for endocytosis and for a low pH-dependent step at an early time point during ALV entry.

Previously, an inhibitory effect of ammonium chloride on ALV entry was not observed (Gilbert et al., 1990). In these studies, ammonium chloride was removed ~6 hr after initiating infection and the effect on virus production was measured several days later (Gilbert et al., 1990). Under these conditions, influenza A, but not ALV-C, virus production was severely reduced, consistent with the conclusion that ALV entered cells by a pH-independent route (Gilbert et al., 1990). However, since our results demonstrate a block to viral entry in the constant presence of ammonium chloride, a likely alternative explanation for these results is that ALV entry was stalled in the presence of ammonium chloride, but resumed when it was removed. Indeed, by washing out ammonium chloride at different time points after beginning infection, we demonstrate that this is the case (Figure 2). These data show that ALV-A and ALV-B are stable for at least 6 hr in treated cells and that entry occurs only when endosomal acidification is reestablished by the removal of ammonium chloride.

The Bafilomycin Block Is Overcome at Low pH and Its Effect Maps to ALV Env

To verify that the defect imposed by these inhibitors was due to a failure of acidification, we asked whether the block by bafilomycin could be overcome by incubating cells with bound virus at low pH. Virus–cell complexes were formed on ice in the presence of bafilomycin, and then transferred directly to a pH 5.0 buffer and incubated at 37°C. These conditions did not lead to efficient restoration of infection, even after a 30 min incubation (Panels 3 and 4, Figure 3A), possibly because they interfere with endocytosis (Hansen et al., 1993). However, infection was efficiently restored at 37°C when virus–cell complexes were incubated at pH 7.4 for 15 min to permit endocytosis before reducing the pH to 5.0 (Panels 5 and 6, Figure 3A). These results suggest the existence of a block to ALV infection when fusion is induced by low pH at the plasma membrane. A similar block to replication has been observed when two endocytosis-dependent viruses, Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV), were induced to fuse with the plasma membrane (Marsh and Bron, 1997).

The bafilomycin block was efficiently overcome at pH 5.5 or below (Figure 3B). Because these experiments were performed in the continued presence of bafilomycin, they rule out the trivial possibility that this inhibitor interferes nonspecifically with reverse transcription. Taken together, these data are consistent with the conclusion that low pH plays a role during ALV entry at a step before reverse transcription, either at the level of Env-dependent membrane fusion or uncoating of viral nucleocapsids.

To determine if low pH acts on the viral envelope protein, we took advantage of the fact that ecotropic murine leukemia virus (MLV) infection of DF-1 cells that express the MCAT-1 receptor (Albritton et al., 1989) is not inhibited by bafilomycin (Figure 3C). This difference in behavior between ALV and MLV is due to properties associated with their envelope proteins. Bafilomycin blocked infection by pseudotyped MLV viruses bearing ALV Env, just as it blocked infection by MLV pseudotypes bearing the classical pH-dependent vesicular stomatitis virus (VSV) G protein (Figure 3C). Reciprocally, ALV pseudotypes with ecotropic MLV Env were resistant to bafilomycin (Figure 3C). Control experiments demonstrated that expression of MCAT-1 in DF-1 cells did not affect the low pH requirement for ALV entry (data not shown). These data demonstrate that low pH is required for activation of the ALV Env during entry, but not for uncoating of the ALV nucleocapsid.

Syncytia Formation by ALV Env-Receptor Complexes Is Low pH Dependent

To test the membrane fusion activity of ALV Env at low pH, a cell–cell fusion assay was used. Uninfected chicken DF-1 cells labeled with octadecylrhodamine (R18) were mixed together with ALV-B infected DF-1 cells expressing EnvB and the green fluorescent protein (GFP). Cell–cell fusion occurred inefficiently, if at all, at neutral pH (Figure 4A), and was equally inefficient when the mixed cell population was treated at pH 5.0 for a short time period (90 s; data not shown), consistent with published results (Gilbert et al., 1990). However,

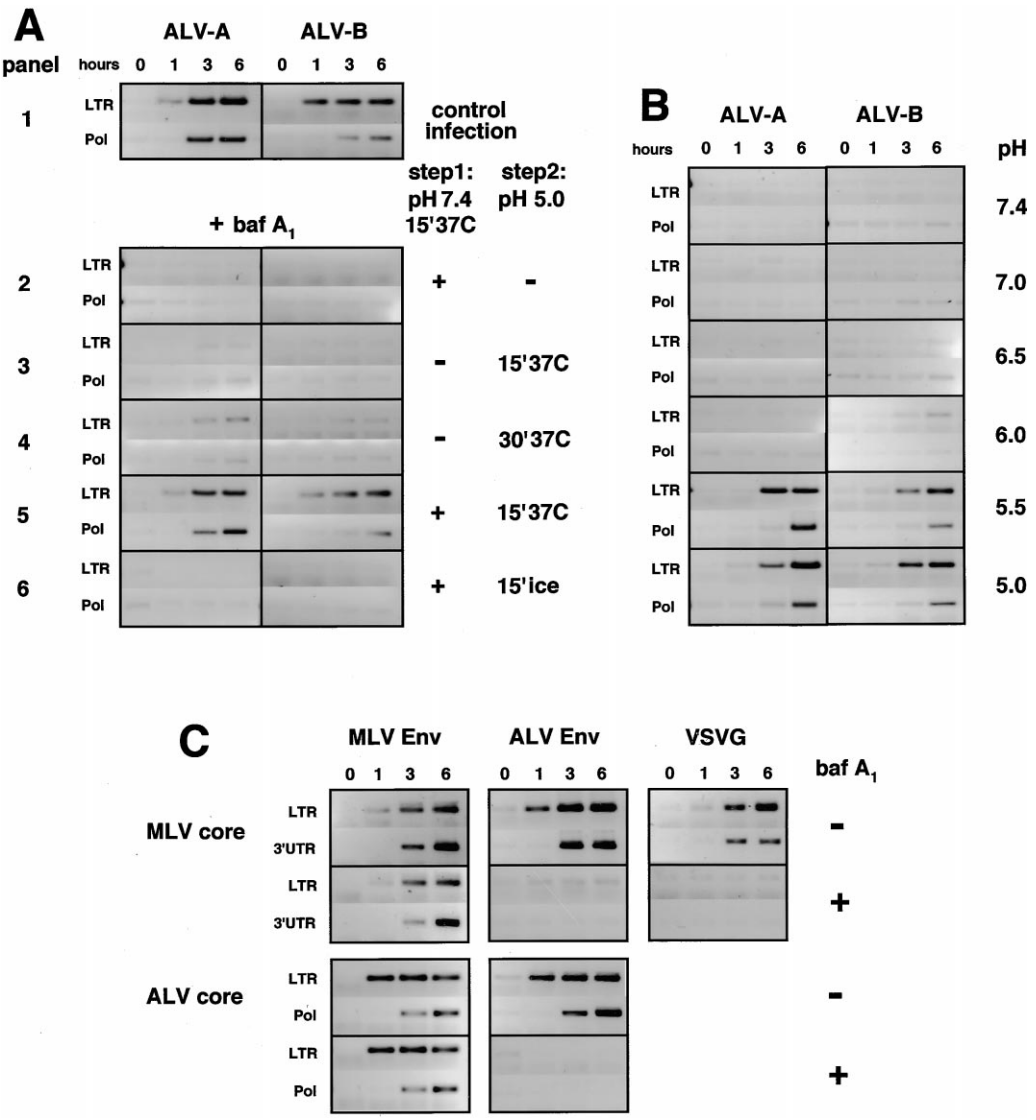


Figure 3. The Bafilomycin Effect Is Overcome at Low pH and Maps to the ALV Env Protein

(A) ALV bound to untreated (panel 1) or bafilomycin-treated DF-1 cells (lower panels) were treated as indicated (step 1 and step 2) before infection was continued in tissue culture and analyzed as described in Figure 1B. Bafilomycin was present throughout the experiment shown in the lower panels.

(B) The pH of the second incubation step shown in Panel 5 of Figure 3A was varied (pH 7.4 to 5.0).

(C) The bafilomycin sensitivity of ALV entry maps to the envelope protein. The infection of DF-1 cells was performed with pseudotyped MLV and ALV viruses (MLV and ALV core) bearing different envelope proteins (ecotropic MLV = MLV Env, ALV Env of subgroup A, B, and E = ALV Env, or VSV-G). For experiments with ecotropic MLV Env, DF-1 cells were used that express MCAT-1 receptor. A different set of primers was used to detect MLV DNA, one for the LTR region (LTR), and a second for the 3′ untranslated region (3′UTR) located downstream of the GFP reporter gene in plasmid pMMP-LTR-GFP.

we found that extending the period of low pH treatment of these cell cultures to between 5 and 30 min led to cell–cell fusion and giant syncytia formation (Figure 4A). Thus, the ALV Env protein induces low pH–dependent cell–cell fusion, albeit perhaps with slower kinetics than is seen with influenza A virus HA.

A requirement for receptor interaction in the low pH–dependent cell–cell fusion mediated by ALV Env became evident in experiments that involved coculturing transfected human 293 cells expressing different combinations of ALV Env proteins and receptors. Low pH–dependent syncytium formation was observed between cells that expressed matched ALV Env-receptor pairs (Figure 4B). In contrast, no syncytia were observed with cells expressing mismatched Env-receptor pairs (Figure 4B), even when the cells were plated at high density (data not shown). These data demonstrate that ALV Env proteins can efficiently fuse cells together in a receptor-dependent and low pH–dependent manner.

Low pH Treatment Rapidly Inactivates ALV Viruses that Are Preloaded with Receptor

Low pH pretreatment does not abolish ALV infectivity under conditions where influenza A virus is inactivated

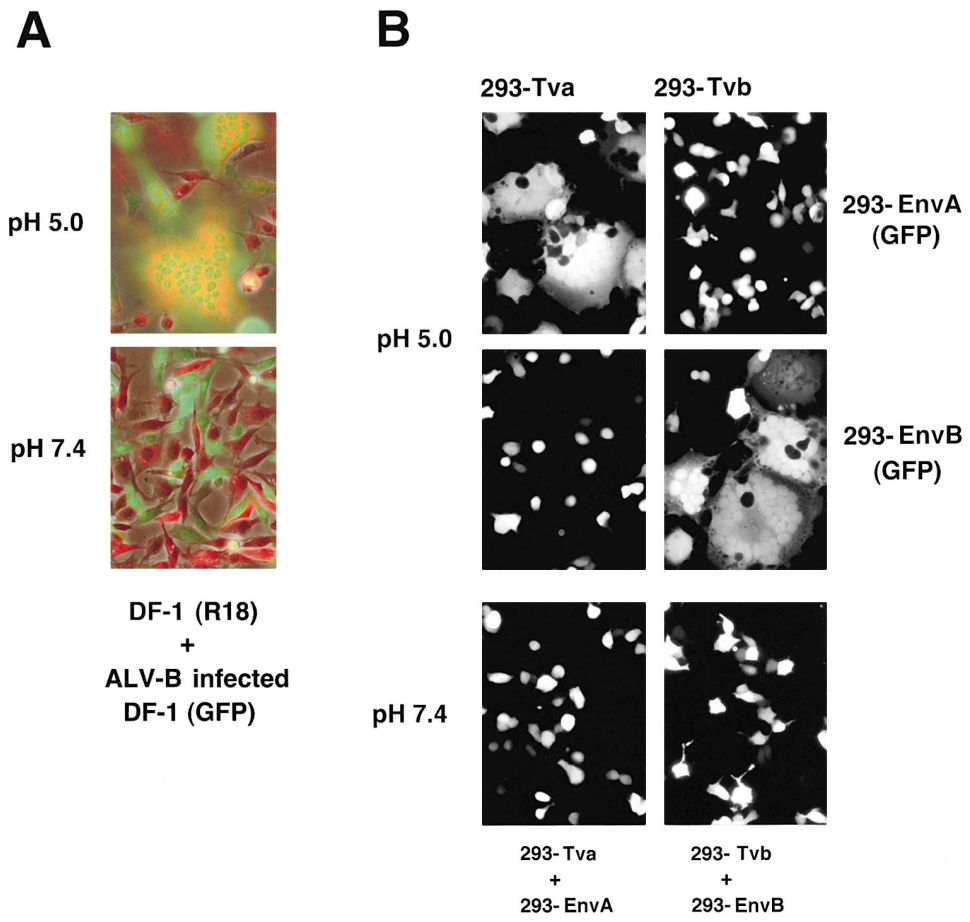


Figure 4. Syncytia Induced by ALV Env-Receptor Interactions and Low pH

(A) Syncytium formation with DF-1 cells. Uninfected DF-1 cells labeled with R18 were mixed with DF-1 cells chronically infected with an ALV-B encoding GFP. These cells were incubated for 30 min at 37°C either at pH 5.0 or pH 7.4 and then with medium at 37°C. After 4 hr incubation, three pictures were taken by fluorescence microscopy with FITC and rhodamine filters and visible light and all three were overlaid.

(B) Syncytium formation with human 293 cells. Transfected 293 cells that express Tva or Tvb were mixed with transfected 293 cells expressing GFP and EnvA or EnvB. Cell mixtures were treated at a pH of 5.0 or 7.4 as in (A) and images were recorded with an FITC filter.

due to premature activation of HA (Gilbert et al., 1990) (Figure 5A). This finding was interpreted to support a pH-independent entry mechanism for ALV (Gilbert et al., 1990). However, in light of the receptor requirement for low pH–dependent cell–cell fusion (Figure 4), we were interested to know whether low pH can inactivate ALV in the presence of receptor. To test this, we utilized soluble receptor-EGF bridge proteins that allow infection of cells expressing EGF receptor (EGFR) (Snitkovsky and Young, 1998; Boerger et al., 1999). This system allows us to manipulate virus/receptor complexes in the absence of cells and test the effects of these treatments on viral infectivity. ALV-A and ALV-B viruses preloaded with Tva-EGF and Tvb-EGF bridge proteins, respectively, were almost as stable at 37°C, as they were at 4°C, when incubated for 6 hr and then titered on cells that express $EGFR^{K721M}$ (M5 cells) (Figure 5B) (Boerger et al., 1999). Indeed, these receptor-loaded viruses were essentially as stable as native virions (Figure 5B). These data demonstrate that neither low pH, nor receptor binding alone, are sufficient to inactivate ALV.

In contrast, virus infectivity was severely abrogated when viruses preloaded with the bridge proteins were incubated for 30 min at pH 5.0 before addition to cells expressing $EGFR^{K721M}$ (Figure 5C). Since these viruses were also unable to infect DF-1 cells (data not shown), these results cannot be explained by pH-dependent dissociation of the bridge proteins from virions. Abrogation of ALV infectivity was observed either with or without a 37°C preincubation step before low pH exposure (Figure 5C) and proceeded within seconds (Figure 5D). The temperature of the low pH treatment influenced the extent of virus inactivation since viral infectivity was only partially reduced when preloaded virions were incubated at low pH on ice (Figure 5C). These data are compatible with a model in which receptor interaction renders ALV Env proteins sensitive to temperature-regulated, low pH-induced activation.

The Combination of Receptor and Low pH Leads to Formation of a Highly Stable Oligomeric TM Complex

To directly test whether receptor and low pH activate ALV Env, we employed a biochemical assay developed

A

pH	7.4	5.0
ALV-A	100 ± 15	78 ± 10.5
ALV-B	100 ± 8.5	59 ± 6

B

Tva/Tvb-EGF	-	-	+	+
temperature	ice	37°C	ice	37°C
ALV-A	100 ± 16	72 ± 10	100 ± 17	65 ± 7
ALV-B	100 ± 5	100 ± 7	100 ± 16	82 ± 9

C

step 1 (preincubation)	37°C	37°C	ice	37°C
step 2 (pH 5.0)	-	37°C	37°C	ice
ALV-A/Tva-EGF	100 ± 12	0.2 ± 0.2	0.6 ± 0.5	24 ± 4
ALV-B/Tvb-EGF	100 ± 14.5	0	0	8.5 ± 0.5

D

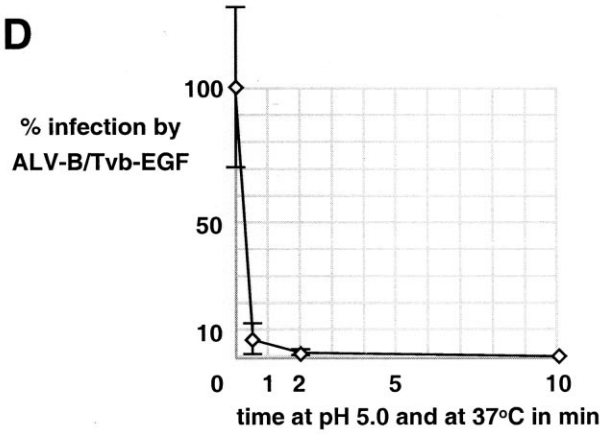


Figure 5. Inactivation of Receptor-Loaded ALV Viruses by Low pH

(A) Native virions are acid stable. ALV virions encoding alkaline phosphatase (AP) were first incubated for 30 min at 37°C and then incubated for an additional 30 min at a pH of 7.4 or 5.0 before addition to cells. Infection was scored by the detection of AP-positive cells two days later. 100% infection corresponds to ~200 and 400 AP-positive cells obtained with neutral pH-treated ALV-A and ALV-B, respectively. The standard deviation of three independent experiments is given next to the average numbers obtained.

(B) Viruses preloaded with receptor are stable at 37°C. ALV viruses encoding GFP were preloaded with Tva-EGF (ALV-A) or Tvb-EGF (ALV-B). Preloaded viruses and native virions were then incubated on ice or at 37°C for six hours. Viruses were then added to 293-Tva cells (ALV-A), 293-Tvb cells (ALV-B), or M5 cells expressing $EGFR^{K721M}$ (preloaded virions). Infected cells expressing GFP were counted by flow cytometry two days later. These numbers were normalized so that the values obtained with the samples kept on ice represent 100% infection. 100% infection corresponds to about 1000 infected cells out of 5000 counted.

(C) Low pH inactivates receptor-loaded virions. Preloaded ALV viruses encoding alkaline phosphatase (AP) were first incubated for 30 min at 37°C or kept on ice. A second 30 min incubation with or without a low pH treatment was carried out either at 37°C or on ice before addition to M5 cells. 100% infection corresponds to about 700 alkaline phosphatase positive cells detected with preloaded viruses treated at neutral pH.

(D) Inactivation kinetics at low pH of preloaded ALV. The incubation time of the low pH treatment at 37°C of the experiment shown in (C) was varied.

by Paul Bates's laboratory to study the oligomeric state of the TM protein. ALV-A TM isolated from virions in a buffer containing 1% SDS migrated as a monomer with a molecular weight of 30 kDa in a reducing SDS-containing polyacrylamide gel (Figure 6A, lane 1). Similarly, ALV-A TM proteins isolated from low pH–treated native virions, or from receptor-loaded virions incubated at neutral pH, were also predominantly monomeric (Figure 6A, lanes 2 and 3). However, following a low pH treatment of receptor-loaded virions at 37°C, high molecular weight forms of TM, including a predominant 70 kDa form were observed (Figure 6A, lane 4). A low pH treatment on ice resulted in inefficient conversion of ALV-A TM in receptor-loaded virions (Figure 6A, lane 5). Similar results were obtained when viruses were preloaded with a soluble Tva protein produced from insect cells (Balliet et al., 1999) instead of Tva-EGF (data not shown). Overall, there was a close correlation between the conditions required for virus inactivation and for formation of the high molecular weight forms of ALV-A TM (compare Figures 6A and 5C).

Analysis of ALV-B yielded similar results with the combination of receptor binding and low pH being needed for complete conversion of TM to higher molecular weight forms at 37°C (Figure 6A, lanes 9–11). However, in this case, a large proportion of the TM protein was also converted by low pH treatment alone (Figure 6A, lanes 7 and 8). These properties are more consistent with the behavior of classical pH-dependent viral glycoproteins. However, since this pH treatment has only a marginal effect on virus infectivity (Figure 5C), these results suggest that the remaining monomeric form of TM (lane 8, Figure 6A) is functional for viral entry.

The conversion of TM on receptor-loaded ALV virions occurred rapidly (within seconds) after the low pH treatment (Figure 6B for ALV-B and data not shown for ALV-A), and was observed most efficiently at pH 5.5 or below (Figure 6C for ALV-B), in agreement with the conditions required to efficiently overcome the block by bafilomycin (Figure 3B).

To determine whether the 70 kDa and higher molecular weight forms of TM that result from receptor interac-

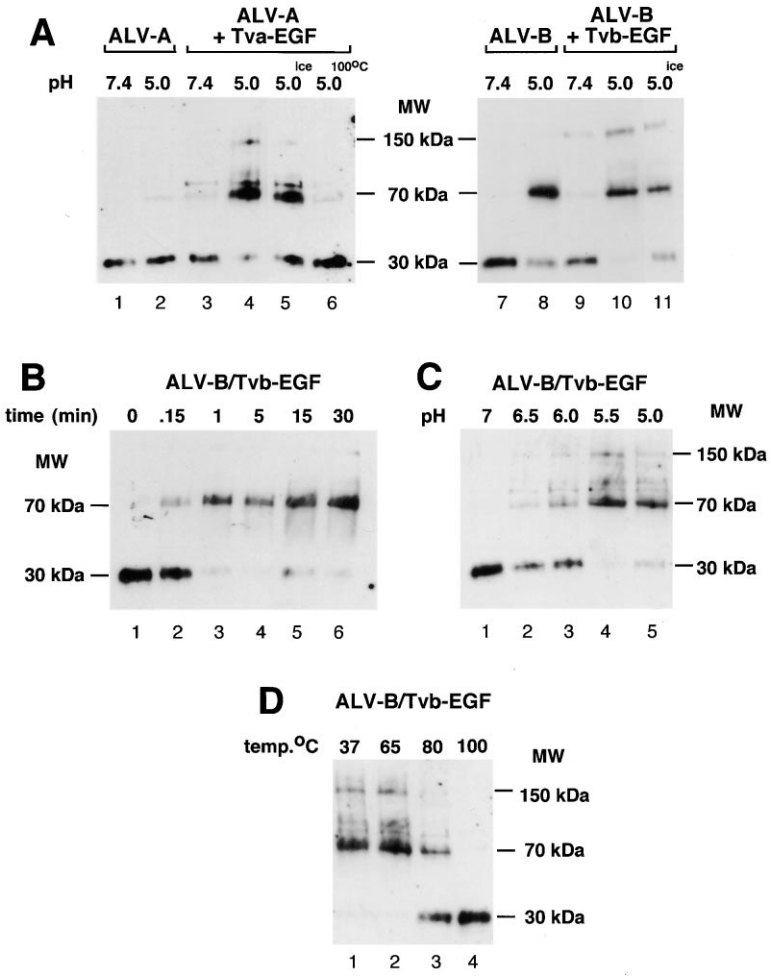


Figure 6. Formation of TM Oligomers on Viruses in Response to Soluble Receptor and Low pH

(A) Conversion of monomeric TM to high molecular weight species after low pH treatment of receptor-loaded viruses. Left panel: ALV-A was concentrated in the absence or presence of Tva-EGF and incubated for 30 min at the indicated pH at 37°C or on ice (ice). Samples were then incubated in 1% SDS sample buffer at 37°C for 5 min before separation on a 4%–15% SDS-containing polyacrylamide gel. An aliquot of the sample shown in lane 4 was boiled (100°C) before loading onto the gel (lane 6). TM was detected by immunoblotting with an antibody specific for its cytoplasmic tail domain. Right panel: The same experiment was performed with ALV-B and Tvb-EGF (lanes 7–11).

(B) ALV-B TM conversion occurs rapidly. An experiment as in (A, lane 10) was performed in which the time of low pH treatment was varied.

(C) ALV-B TM is converted most efficiently at pH 5.5 and below. An experiment as in (A, lane 10) was performed with the exception that buffers of decreasing pH values were used.

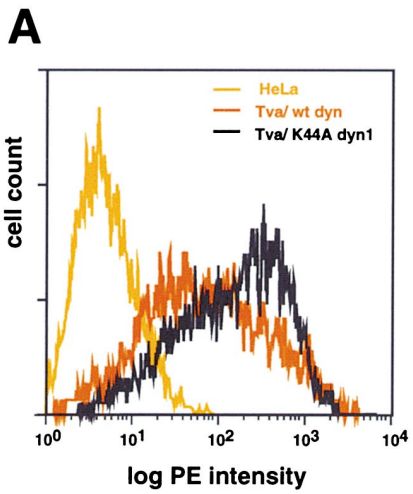
(D) The high molecular weight protein species are highly stable ALV-B TM oligomers. An experiment as in (A, lane 10) was carried out except that prior to electrophoresis, the samples were incubated in 1% SDS at 37, 65, 80, and 100°C.

tion with native ALV virions and low pH are stable oligomeric forms of TM, these complexes were incubated in SDS-containing buffers at different temperatures prior to gel electrophoresis. The high molecular weight forms of TM were converted back to the 30 kDa monomeric form by incubation at 100°C (Figure 6A, lane 6 for ALV-A and Figure 6D, lane 4 for ALV-B), but were stable at 37°C or 65°C (Figure 6D for ALV-B). The apparent melting temperature of these high molecular weight forms of TM was ~80°C (Figure 6D, lane 3 for ALV-B). These data suggest that the 70 kDa and higher molecular weight proteins of TM that are generated from native ALV virions in response to soluble receptor binding and low pH are highly stable oligomeric forms of the viral glycoprotein, similar to those observed for other fusion-activated viral glycoproteins (Weissenhorn et al., 1999).

ALV Entry Is Reduced in Cells Expressing Dominant-Negative Dynamin

The effect of lysosomotropic agents and the low pH requirement for ALV entry is consistent with a model in which this virus is internalized by receptor-mediated endocytosis and then trafficked to acidic endosomes where viral fusion occurs. Two major internalization pathways, clathrin-mediated endocytosis, and that occurring through caveolae are, at least in part, mediated by dynamin (Damke et al., 1994; Oh et al., 1998). To ask whether or not dynamin is involved in ALV uptake, HeLa cells were transfected with plasmids encoding either wild-type or dominant-negative (K44A) dynamin along with those encoding the Tva receptor and GFP. Transfected, GFP-expressing cells were then sorted by flow cytometry. Analysis of the level of Tva expression on the surfaces of these cells demonstrated an upregulation of Tva caused by the mutant dynamin, a phenotype consistent with a role for dynamin in the internalization of this receptor (Figure 7A). The transfected cells were then challenged with ALV-A and subsequent viral DNA synthesis was monitored using a quantitative TaqMan-based, real-time PCR assay, which allows for detection of modest differences in the level of viral entry.

A 60% to 80% inhibition of ALV-A entry was observed in cells expressing mutant dynamin (Figure 7B), even though these cells had upregulated Tva at their cell surface, as compared with cells expressing wild-type dynamin. The level of dynamin K44A-induced inhibition of ALV entry is similar to that seen with a known pH-dependent virus, Semliki Forest virus (SFV) (DeTuello and Kirchhausen, 1998) and for the inhibition of receptor-mediated EGF uptake (Damke et al., 1994). The observed residual infection may be due the induction of alternative uptake pathways in cells expressing the mu-



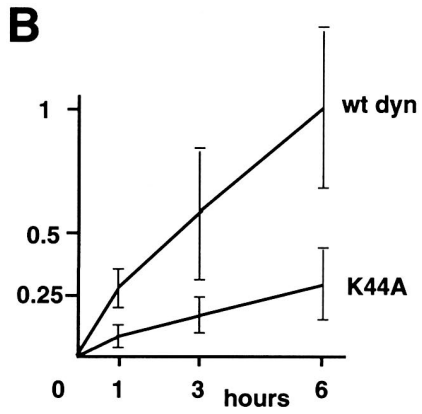


Figure 7. Reduced ALV-A Entry into Cells Expressing Dominant-Negative Dynamin

(A) Tva surface expression as measured by flow cytometry of HeLa cells expressing wt or dominant-negative dynamin.

(B) HeLa cells expressing Tva and either wild-type (wt dyn) or dominant-negative dynamin (K44A) were infected with ALV-A, and DNA from these cells was prepared for quantitative, real-time PCR analysis to measure the relative level of early viral DNA products. The results from 5 independent experiments are shown. The amount of early viral DNA products produced at the 6 hr time point in the presence of wild-type dynamin is represented by a relative value of 1.0.

tant dynamin (Damke et al., 1995). These results thus support a role of dynamin during ALV entry.

Discussion

In this report, we demonstrate that avian leukosis virus (ALV) uses a pH-dependent entry mechanism, and not a pH-independent mode as previously thought. These results indicate that ALV Env is activated by a specific Env-receptor interaction that renders the viral glycoprotein sensitive to low pH-induced activation. These biochemical requirements for ALV Env activation must correlate in cells with viral uptake by endocytosis followed by trafficking to a low pH endosome where fusion occurs. Several independent lines of evidence support this model. An essential low pH step is evident from the inhibition of ALV entry by lysosomotropic agents and the ability of low pH: (1) to overcome the bafilomycin block; (2) to cause syncytia; (3) to inactivate receptor-loaded viruses; and (4) to induce the formation of TM oligomers that have properties similar to those of other fusion-activated viral glycoproteins. Furthermore at low pH, ALV receptors were required for syncytia formation, for virus inactivation, and for TM oligomer formation. A role for endocytosis is supported by the inhibition of ALV entry by lysosomotropic agents. The ionophores monensin and nigericin act by dissipating proton gradients across intracellular membranes and would not inhibit fusion at the plasma membrane. The pH of 5.5 that is required to overcome the block imposed by bafilomycin is characteristic of a late endosomal or lysosomal compartment and is not observed at the plasma membrane of cultured fibroblasts. Consistently, ALV does not cause syncytia in tissue culture at neutral pH (Gilbert et al., 1990; this paper). Finally, ALV entry was reduced by dominant-negative dynamin, suggesting that dynamin-mediated endocytosis contributes to the internalization mechanism of ALV.

A novel and unanticipated principle revealed by our studies on ALV entry is that receptor interaction can convert a viral envelope protein from a native metastable state that is insensitive to low pH to a second metastable state that is sensitive to low pH for membrane fusion. Consistent with the existence of a long-lived Env-receptor intermediate, ALV preloaded with receptor-ligand bridge proteins were very stable at 37°C. Furthermore, ALV bound to cellular receptors was similarly stable when acidification of cellular compartments was inhibited. ALV receptors may induce conformational changes in Env that render the viral glycoprotein more sensitive to the second low pH step. Several conformational changes in EnvA that have been described in the presence of soluble receptor may be due to receptor priming rather than complete activation as previously thought (Gilbert et al., 1995; Hernandez et al., 1997; Damico et al., 1998). It is also possible that a component of the low pH sensor resides within the bound receptor and if so, low pH–induced conformational changes in the receptor might trigger fusion-relevant changes in Env.

The receptor dependence of the low pH effect on ALV entry, coupled with the stability of ALV-receptor complexes in ammonium chloride–treated cells explain why the entry mechanism used by this virus was previously interpreted to be pH-independent (Gilbert et al., 1990). In contrast to classical pH-dependent viruses such as influenza A virus, ALV is resistant to low pH in the absence of receptor (Gilbert et al., 1990; Puri et al., 1990). Also, unlike influenza A virus, ALV is stable in ammonium chloride–treated cells for 6 hr, explaining why this treatment did not have a detectable effect on viral entry as measured by virus production several days later (Gilbert et al., 1990). In addition, a brief low pH treatment of CEFs under conditions that caused HA-dependent cell–cell fusion, is not sufficient for ALV Env/receptor–dependent syncytia formation (Gilbert et al., 1990 and data not shown). It remains to be explained why the continued presence of ammonium chloride had

no apparent effect upon subgroup C ALV Env–dependent membrane fusion as measured by octadecylrhodamine (R18) transfer (Gilbert et al., 1990). Although this result might be explained by subtype-specific viral differences and by spontaneous exchange of R18 (Stegmann et al., 1995), these data could indicate that receptor-induced conformational changes in ALV Env may lead to the formation of a fusion intermediate allowing partial lipid exchange, but not complete fusion (Chernomordik et al., 1999).

Previously, it was suggested that certain retroviruses may use a pH-dependent mechanism for cell entry (Andersen and Nexo, 1983; Redmond et al., 1984). Based upon established classification criteria, the glycoproteins of these viruses behaved exactly like those of a classical pH-dependent virus, i.e., they were predicted to be activated for fusion by low pH alone (Redmond et al., 1984). However, in light of our observations with ALV, the entry mechanisms used by these viruses, and by other pH-dependent viruses, now need to be reinvestigated to determine whether their cellular receptors also play a critical role in rendering the cognate viral glycoproteins responsive to low pH–induced fusion activation. The entry mechanism described in this paper for ALV also raises the question of whether other "pH-independent" viruses also require a downstream low pH step that may have been similarly overlooked because their behavior was compared to classical pH-dependent viruses. Future work will reveal whether the two-step mechanism of viral entry described in this report is unique to ALV, or instead whether this avian retrovirus is the founding member of a novel group of viruses that need receptor priming and low pH triggering for cell entry.

Experimental Procedures

Materials

Lysosomotropic agents were from Sigma. Chloroquine and NH_4Cl stock solutions were adjusted to pH 7.4 before use. The following oligonucleotide primers were used for seminested PCR amplification: (A) Primers for the detection of ALV LTR and *pol*: ggtggaagtaag gtggtacgatc (U3, sense), gcttcatgcaggtgctcgtagtc (U5, antisense), caatgtggtgaatggtcaaatggc (R, antisense), gcacccgtaggcttggtagcag (pol, sense), cctattgccccctgttcacatc (pol, antisense), acttcagtggctt cgcccagcg (pol, sense). (B) Primers for the detection of MLV reverse transcription products: ctaaccaatcagttcgcttctcgc (U3, sense), acgg gtagtcaatcactcagag (U5, antisense), ctgcaagagggtttattgagaacac (R, antisense), ctgtgtctgtccgattgtctagtg (UTR, sense), ccgtggtggaact gacgag (UTR, sense), gacagagacaacacagaacgatg (UTR, antisense).

Human 293 cells and HeLa cells were from the ATCC, avian DF-1 cells, and M5 cells have been described previously (Himly et al., 1998; Snitkovsky and Young, 1998). Primary chicken embryonic fibroblasts (CEF) were grown at 41°C and used between passage numbers 2 and 5. DF-1 cells expressing MCAT-1 (clone DFJ8) were obtained from Stephen Hughes, NCI Frederick. Stable 293 cell lines expressing Tva (2.1 cells) and Tvb^{S1} (S1-5 cells) were generated by transfection of plasmid pKZ458 (K. Zingler and J. Y., unpublished data) and cotransfection of plasmid pHA1 (Adkins et al., 2000) and plasmid pPur (Clontech). Single cell clones were isoloated with 1 μg/ml puromycin and receptor expression was confirmed by flow cytometry.

Preparation of Viruses and Soluble Bridge Proteins

For ALV-A and ALV-B production, RCASBP(A)-GFP and RCASBP(B)-GFP viruses (Boerkoel et al., 1993; M. van Brocklin and M. Federspiel, personal communication) were produced from chronically infected DF-1 cells and RCASBP(B)-AP virus (Boerkoel et al., 1993) was produced from chronically infected CEFs. The viral titers on 293 cells expressing ALV receptors were 10^6 (RCASBP(A)-GFP), 10^7 (RCASBP(B)-GFP), and 10^4 (RCASBP(B)-AP) infectious units/ml, respectively. Ecotropic MLV (Cl-1; Fan and Paskind, 1974) and ALV containing the ecotropic MLV envelope protein (RCASBP(eco); Stephen Hughes) were harvested from the supernatant of chronically infected NIH3T3 and DFJ8 cells, respectively.

MLV pseudotyped viruses were generated using a tripartite transfection system (Boerger et al., 1999). Typically, an 80% confluent 10 cm plate of 293 cells was transfected with 15 μg plasmid pMD.old.gag.pol, 20 μg of plasmid pMMP-LTR-GFP, and 5 μg of plasmids encoding EnvA, EnvB, and EnvE (pAB6, pAB7, and pAB 9, Boerger et al., 1999) and VSV-G (pMD.G, a modified version of pMDtet.G [Ory et al., 1996]). The medium was changed after transfection and the cells were incubated for an additional 6 hr with 1 mg/ml DNase I (Boehringer Mannheim) to remove excess plasmid DNA. Viruses containing supernatants were harvested 48 and 60 hr posttransfection and stored at −80°C. Before use, the virus was again incubated with 0.1 mg/ml DNase I for one hour at room temperature.

The Tva-EGF and Tvb-EGF bridge proteins were produced from 293 cells as described previously (Snitkovsky and Young, 1998; Boerger et al., 1999). Soluble Tva prepared from insect cells (Balliet et al., 1999) was a gift from Paul Bates.

PCR Entry Assays

Cells were detached from the plate with phosphate-buffered saline (PBS)/5mM EDTA, pelleted at 1000 × g, and washed twice with cold PBS. Approximately 10^6 infectious units of virus were added to 10^6 cells on ice and then mixed continuously for one hour at 4°C. Virus/cell complexes were pelleted and washed once to remove unbound virus, then split into 4 aliquots. Total DNA from one aliquot was immediately prepared for analysis, and the remaining three samples were seeded in 12 well plates and incubated with medium in a 5% CO_2 incubator at 37°C or 41°C (CEF) for 1, 3, or 6 hr to initiate infection. At each time point, cells were removed from the plate with PBS/EDTA, counted, pelleted, and incubated for 15 min at room temperature in 0.3 ml of a buffer containing 5 mM EDTA/130 mM salt/100 μg proteinase K/1% SDS. All samples were phenol/chloroform extracted twice and the DNA was precipitated and resuspended in water at a concentration equivalent to 5000 cells per μl.

For seminested PCR amplifications, 1 μl of each DNA sample was used for a standard PCR reaction (30 s 94°C; 40 s 65°C; 1 min 15 s 72°C). After the first round of amplification (15 cycles), one-fifth of the products were used for the second seminested round (18 cycles). Approximately one-fourth of the PCR products were separated on 2.5% agarose gels containing ethidum bromide and visualized using a Biorad FluorS Imager. These PCR conditions were selected because they allowed semiquantitive detection of control proviral DNA molecules over a range of three logs ($\sim 10^3$ to 10^6). To study the effects of lysosomotropic agents on viral entry, cells were incubated with the inhibitors for about one hour before virus binding and then kept in the continued presence of the drugs unless otherwise indicated.

To analyze the effect of dominant-negative dynamin, an 80% confluent 10 cm plate of HeLa cells was transiently transfected using Superfect (Qiagen) with 3 μg of plasmid pCB6 0.95 encoding Tva (Bates et al., 1993) and 15 μg of pcDNA based plasmids (Invitrogen) encoding wt or dominant-negative dynamin 1, respectively (Damke et al., 1994; Sever et al., 1999; S. Schmid, personal communication), and with 2 μg of plasmid pEGFP-N1 (Clontech) encoding GFP. Forty hours later, transfected, GFP-expressing cells were sorted by flow cytometry, and one half was challenged with ALV-A (as described above) and the other half was prepared for flow cytometry using SuA-IgG (Brojatsch et al., 1996) and a PE-conjugated antibody specific for rabbit immunoglobulins (Jackson IR). The real-time PCR experiments to detect early viral DNA products were performed in a final volume of 50 μl in 1 × Universal PCR Master Mix (Perkin Elmer) using the DNA template from 5000 cell equivalents and 200 nM each of the following oligonucleotides: 5′-ACTGAATTCCGCA TTGCAGAG-3′ (sense); 5′-CCATCAACCCAGGTGCACA-3′ (antisense); and 5′-TGCCTAGCTCGATACAATAAACGCCATTTG (Taqman probe, Perkin Elmer). The PCR analysis (50°C 2 min, 95°C 10 min, [95°C 15 s; 60°C 1 min] × 40 cycles) was performed with an ABI 7700 instrument.

Syncytium Formation

To study cell–cell fusion, R18 (Molecular Probes) was added to a 10 cm plate of uninfected DF-1 cells at a final concentration of 1:50,000. The cells were collected with PBS/ EDTA one hour later, pelleted, and washed with PBS and then mixed at a 1:1 ratio with DF-1 cells chronically infected with RCASBP(B)-GFP. Three hours later, the cells were washed, incubated for 30 min at 37°C with PBS adjusted with HCl to pH 7.4 or pH 5.0 (identical results were obtained when PBS pH 5.0 was supplemented with 10 mM acetate). Images were recorded with an inverted Nikon microscope (Nikon eclipse TE 300) after a subsequent 4 hr incubation in medium at 37°C. For DF-1 cells, images recorded with visual light, FITC, and rhodamine filters were overlaid and recolored using IPLab Spectrum P-software. In similar experiments, 293 cells transiently transfected with 20 μg GFP (pEGFP-N1, Clontech) and 5 μg pAB6 (EnvA) or pAB7 (EnvB) were tested for their ability to undergo pH-dependent syncytium formation with 293 2.1 cells (Tva) and 293 S1–5 cells (TvbS1).

Virus Inactivation

Native virions and Tva-EGF/Tvb-EGF preloaded viruses were concentrated and purified essentially as described elsewhere (Boerger et al., 1999) and kept on ice until used. To alter the pH, virions were diluted 100-fold in medium that was kept at pH 7.4 or adjusted to pH 5.0 with HCl. The samples were then incubated either on ice or at 37°C for 30 min prior to neutralization with an equal volume of medium buffered with 25mM HEPES (pH 7.4). The virus was then added to cells in 12-well plates and 16 hr later the medium was replaced. Approximately 56 hr later, infected cells were identified either by heat stable alkaline phosphatase expression (NBT/BCIP, GibcoBRL) or by flow cytometry. All experiments were performed in triplicate.

TM Oligomer Formation

Tva-EGF and Tvb-EGF preloaded virions were generated by mixing 8 ml of RCASBP(A)-GFP or 14 ml RCASBP(B)-GFP virus-containing supernatants with 20 ml or 14 ml of bridge protein–containing supernatants, respectively. The preloaded viruses, as well as native viruses, were then purified and concentrated by ultracentrifugation in a SW28 rotor for 1.5 hr at 25 K, and resuspended overnight in 150 μl of Tris (pH 8)/130mM saline/1mM EDTA (TNE). Concentrated virus (typically 25 μl or 2 μl for ALV-A and ALV-B, respectively) was diluted into a volume of 40 μl PBS. To alter the pH, samples were adjusted to 7.4, 7.0, 6.5, 6.0, 5.5, or 5.0 in a final volume of 50 μl with a predetermined amount of HEPES buffer (pH 5.0 and pH 3.0). The final HEPES concentration necessary to maintain the indicated pH varied between 50 mM and 70 mM. Alternatively, the pH was adjusted using Tris/acetate (pH 4.5). The samples were then incubated either on ice or at 37°C for 30 min prior to neutralization with 2 μl of 1 M Tris (pH 8.0). The samples were incubated at 37°C for five minutes in a buffer containing β-mercaptoethanol and a final concentration of 1% SDS (and at 65, 80, and 100°C for analysis of TM oligomers) before electrophoresis on a 4%–15% gradient gel containing SDS (Biorad). TM proteins were analyzed by Western blotting and enhanced chemiluminescence with an antibody specific for the C terminus of the TM subunit of ALV Env (CGQPESRIV) in TBS/0.1%Tween20/1%BSA.

Acknowledgments

We are grateful to John Balliet and Paul Bates for sharing unpublished results and for their generous support and help with the TM oligomer formation assay that they had developed. We thank Joan Bruegge and John Daley for assistance and Ken Bradley, Sophie Snitkovsky, Heather Adkins, Stephen Hughes, Tomas Kirchhausen, Sandra Schmid, R. Mulligan, and M. Federspiel for cell lines and reagents. We are especially indebted to Judith White for extensive discussion and also to Kent Matlack, Kathrin Plath, and Anna Barnett for critical reading of the manuscript. This work was supported by NIH grant CA70810 (to J. Y.). J. C. is a Howard Hughes Medical Institute Investigator and W. M. is a Research Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

Received December 7, 1999; revised September 6, 2000.

References

Adkins, H.B., Brojatsch, J., and Young, J.A. (2000). Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. *74*, 3572–3578.

Albritton, L.M., Tseng, L., Scadden, D., and Cunningham, J.M. (1989). A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell *57*, 659–666.

Andersen, K.B., and Nexo, B.A. (1983). Entry of murine retrovirus into mouse fibroblasts. Virology *125*, 85–98.

Balliet, J.W., Berson, J., D'Cruz, C.M., Huang, J., Crane, J., Gilbert, J.M., and Bates, P. (1999). Production and characterization of a soluble, active form of Tva, the subgroup A avian sarcoma and leukosis virus receptor. J. Virol. *73*, 3054–3061.

Bates, P., Young, J.A., and Varmus, H.E. (1993). A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell *74*, 1043–1051.

Boerger, A.L., Snitkovsky, S., and Young, J.A. (1999). Retroviral vectors preloaded with a viral receptor-ligand bridge protein are targeted to specific cell types. Proc. Natl. Acad. Sci. USA *96*, 9867–9872.

Boerkoel, C.F., Federspiel, M.J., Salter, D.W., Payne, W., Crittenden, L.B., Kung, H.J., and Hughes, S.H. (1993). A new defective retroviral vector system based on the Bryan strain of Rous sarcoma virus. Virology *195*, 669–679.

Brojatsch, J., Naughton, J., Rolls, M.M., Zingler, K., and Young, J.A. (1996). CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. Cell *87*, 845–855.

Bullough, P.A., Hughson, F.M., Skehel, J.J., and Wiley, D.C. (1994). Structure of influenza haemagglutinin at the pH of membrane fusion. Nature *371*, 37–43.

Carr, C.M., and Kim, P.S. (1993). A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell *73*, 823–832.

Chernomordik, L.V., Leikina, E., Kozlov, M.M., Frolov, V.A., and Zimmerberg, J. (1999). Structural intermediates in influenza haemagglutinin-mediated fusion. Mol. Membr. Biol. *16*, 33–42.

Damico, R.L., Crane, J., and Bates, P. (1998). Receptor-triggered membrane association of a model retroviral glycoprotein. Proc. Natl. Acad. Sci. USA *95*, 2580–2585.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. (1994). Induction of mutant dynamin specifically blocks endocytic vesicle formation. J. Cell Biol. *127*, 915–934.

Damke, H., Baba, T., van der Bliek, A.M., and Schmid, S.L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. J. Cell Biol. *131*, 69–80.

DeTuello, L., and Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. EMBO J. *17*, 4585–4593.

Drose, S., and Altendorf, K. (1997). Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J. Exp. Biol. *200*, 1–8.

Durrer, P., Galli, C., Hoenke, S., Corti, C., Gluck, R., Vorherr, T., and Brunner, J. (1996). H+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region. J. Biol. Chem. *271*, 13417–13421.

Duve, C.D., Barsy, T.D., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974). Lysosomotropic agents. Biochem. Pharmacol. *23*, 2495–2531.

Fan, H., and Paskind, M. (1974). Measurement of the sequence of cloned moloney murine leukemia virus 60 to 70S RNA: evidence for a haploid genome. J. Virol. *14*, 421–429.

Gilbert, J.M., Mason, D., and White, J.M. (1990). Fusion of Rous sarcoma virus with host cells does not require exposure to low pH. J. Virol. *64*, 5106–5113.

Gilbert, J.M., Hernandez, L.D., Balliet, J.W., Bates, P., and White, J.M. (1995). Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J. Virol. *69*, 7410–7415.

Hansen, S.H., Sandvig, K., and van Deurs, B. (1993). Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J. Cell Biol. *121*, 61–72.

Hernandez, L.D., Hoffman, L.R., Wolfsberg, T.G., and White, J.M. (1996). Virus-cell and cell-cell fusion. Annu. Rev. Cell. Dev. Biol. *12*, 627–661.

Hernandez, L.D., Peters, R.J., Delos, S.E., Young, J.A., Agard, D.A., and White, J.M. (1997). Activation of a retroviral membrane fusion protein: soluble receptor-induced liposome binding of the ALSV envelope glycoprotein. J. Cell Biol. *139*, 1455–1464.

Himly, M., Foster, D.N., Bottoli, I., Iacovoni, J.S., and Vogt, P.K. (1998). The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. Virology *248*, 295–304.

Marsh, M., and Bron, R. (1997). SFV infection in CHO cells: cell-type specific restrictions to productive virus entry at the cell surface. J. Cell Sci. *110*, 95–103.

Oh, P., McIntosh, D.P., and Schnitzer, J.E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol. *141*, 101–114.

Ory, D.S., Neugeboren, B.A., and Mulligan, R.C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc. Natl. Acad. Sci. USA *93*, 11400–11406.

Puri, A., Booy, F.P., Doms, R.W., White, J.M., and Blumenthal, R. (1990). Conformational changes and fusion activity of influenza virus hemagglutinin of the H2 and H3 subtypes: effects of acid pretreatment. J. Virol. *64*, 3824–3832.

Redmond, S., Peters, G., and Dickson, C. (1984). Mouse mammary tumor virus can mediate cell fusion at reduced pH. Virology *133*, 393–402.

Sever, S., Muhlberg, A.B., and Schmid, S.L. (1999). Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. Nature *398*, 481–486.

Snitkovsky, S., and Young, J.A. (1998). Cell-specific viral targeting mediated by a soluble retroviral receptor-ligand fusion protein. Proc. Natl. Acad. Sci. USA *95*, 7063–7068.

Sodroski, J.G. (1999). HIV-1 entry inhibitors in the side pocket. Cell *99*, 243–246.

Stegmann, T., White, J.M., and Helenius, A. (1990). Intermediates in influenza induced membrane fusion. Embo J. *9*, 4231–4241.

Stegmann, T., Orsel, J.G., Jamieson, J.D., and Padfield, P.J. (1995). Limitations of the octadecylrhodamine dequenching assay for membrane fusion. Biochem. J. *307*, 875–878.

Telesnitsky, A., and Goff, S.P. (1997). Reverse transcriptase and the generation of retroviral DNA. In Retroviruses, J.M. Coffin, S.H. Hughes and H.E. Varmus, eds. (New York, NY: Cold Spring Harbor Laboratory Press), pp 121–160.

Weissenhorn, W., Dessen, A., Calder, L.J., Harrison, S.C., Skehel, J.J., and Wiley, D.C. (1999). Structural basis for membrane fusion by enveloped viruses. Mol. Memb. Biol. *16*, 3–9.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., and Chen, I.S. (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell *61*, 213–222.