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Mouse mammary tumor virus uses mouse but not human transferrin receptor 1 to reach a low pH compartment and infect cells

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ABSTRACT

Mouse mammary tumor virus (MMTV) is a pH-dependent virus that uses mouse transferrin receptor 1 (TfR1) for entry into cells. Previous studies demonstrated that MMTV could induce pH 5-dependent fusion-from-with of mouse cells. Here we show that the MMTV envelope-mediated cell–cell fusion requires both the entry receptor and low pH (pH 5). Although expression of the MMTV envelope and TfR1 was sufficient to mediate low pH-dependent syncytia formation, virus infection required trafficking to a low pH compartment; infection was independent of cathepsin-mediated proteolysis. Human TfR1 did not support virus infection, although envelope-mediated syncytia formation occurred with human cells after pH 5 treatment and this fusion depended on TfR1 expression. However, although the MMTV envelope bound human TfR1, virus was only internalized and trafficked to a low pH compartment in cells expressing mouse TfR1. Thus, while human TfR1 supported cell–cell fusion, because it was not internalized when bound to MMTV, it did not function as an entry receptor. Our data suggest that MMTV uses TfR1 for all steps of entry: cell attachment, induction of the conformational changes in Env required for membrane fusion and internalization to an appropriate acidic compartment.

Introduction

Infection of mice by mouse mammary tumor virus (MMTV) has long been known to cause mammary tumors in mice. Whether a similar infectious agent exists in humans has been a subject of debate since the discovery of MMTV. There have been reports that continuous passage of MMTV on human breast cancer cell lines resulted in adapted viruses that could infect human cells (Howard and Schlom, 1980; Lasfargues et al., 1979) and more recently, Indik et al. reported infection of several human cell lines with a GFP-marked MMTV(Indik et al., 2005). Additionally, several groups have detected MMTV-like sequences in up to 40% of human breast cancer samples, but not normal tissue (Etkind et al., 2000; Ford et al., 2003; Liu et al., 2001; Pogo et al., 1999; Szabo et al., 2005; Wang et al., 1995; Zammarchi et al., 2006). MMTV-like sequences have also been detected in patients with the autoimmune disease, primary biliary cirrhosis (PBC) (Xu et al., 2003). Other groups, however, have been unable to find such sequences (Bindra et al., 2007; Mant et al., 2004; Selmi et al., 2004).

Mouse transferrin receptor 1 (mTfR1) is the MMTV entry receptor (Ross et al., 2002). Although mouse and human TfR1 (hTfR1) show 76% identity and 86% homology at the amino acid level, we have shown that the human receptor does not serve as an entry receptor. By constructing human/mouse chimeric TfR1 molecules, we mapped the critical amino acids required to support MMTV entry to two adjacent regions in the extra-cellular domain of mouse TfR1 that are distinct from the transferrin binding site (Wang et al., 2006).

Previous studies also demonstrated that MMTV is a pH-dependent virus, since infection can be efficiently blocked by inhibitors that block acidification of intra-cellular compartments (Ross et al., 2002). As exemplified by influenza A virus, virus/cell membrane fusion required for entry of low pH-dependent viruses occurs at the endosomal membrane following receptor-mediated endocytosis (Skehel and Wiley, 2000). Such intra-cellular fusion events can often be mimicked at the cell surface by treating cells expressing viral fusion proteins with low pH buffer, resulting in syncytia formation (Redmond et al., 1984).

We show here that MMTV requires trafficking to a low pH compartment to achieve infection of cells and that this trafficking did not require Eps15, which is known to play a role in clathrin-mediated endocytosis (Benmerah et al., 1999) or the recently identified transferrin receptor transport protein (TTP) (Tosoni et al., 2005). We also show that although MMTV uses mTfR1 and not hTfR1 for infection, low pH induced cell–cell fusion occurred on human cells that express the MMTV envelope (Env) and both Env and hTfR1 were required for this event. However, MMTV was unable to mediate internalization of

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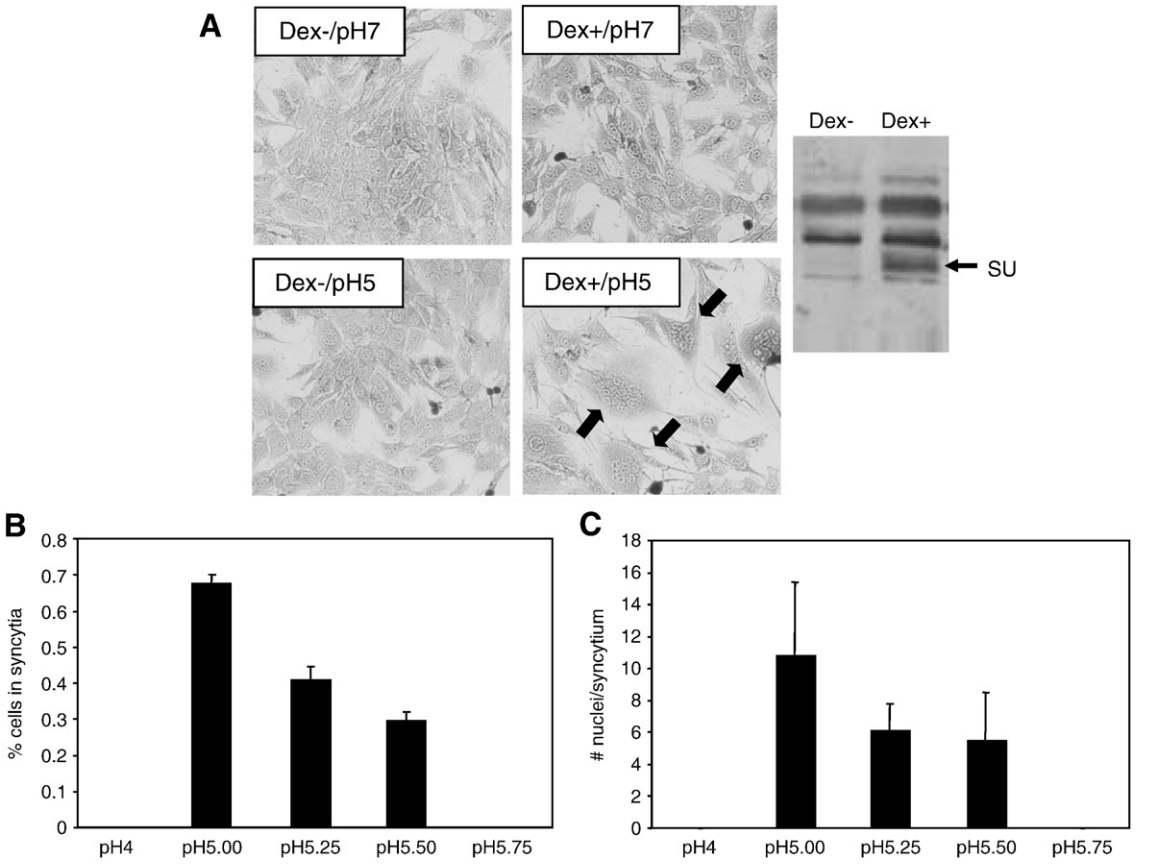


Fig. 1. MMTV induces low pH-dependent cell–cell fusion. (A) Syncytia formation requires MMTV Env expression and low pH. Mm5MT cells were grown in the presence (Dex+) or absence (Dex−) of dexamethasone for 24 h and exposed to pH 5 or pH 7 media. A Western blot of cell extracts from duplicate wells grown in the absence or presence of dexamethasone probed with anti-SU antisera is also shown. (B and C) Maximum cell–cell fusion pH occurs at pH 5. Mm5MT cells induced overnight with dexamethasone were treated with media at the indicated pH. Twenty-four hours later, the cells were stained with Giemsa and the percentage of cells in syncytia (B) and the number of nuclei/syncytium (C) were determined by counting.

hTfR1. Thus, because MMTV requires trafficking to a low pH compartment to enter cells, it was unable to infect human cells despite engaging the hTfR1 receptor. These data provide further evidence that MMTV is unlikely to be a causal agent in human breast cancer.

Results

MMTV induces syncytia formation at low pH

Previously, we showed that MMTV infection required a low pH compartment, since treatment of cells with lysosomotropic agents inhibited infection by viral pseudotypes (Ross et al., 2002). To further examine the pH requirements of MMTV infection, we used a cell–cell fusion assay similar to that described by Redmond et al. with the Mm5MT murine mammary carcinoma cell line, derived from an MMTV-induced mammary tumor (Redmond et al., 1984). Mm5MT cells were grown in the presence or absence of the synthetic glucocorticoid dexamethasone to induce viral protein expression (Fig. 1A) and then treated with pH 5 media for 15 min; we determined experimentally that 15 min incubation gave the highest levels of fusion (not shown). Only dexamethasone-induced cells expressing high levels of Env and treated with pH 5 media showed syncytia

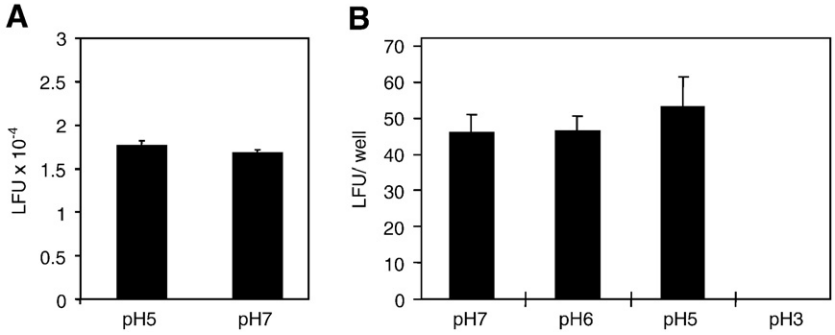


Fig. 2. Low pH treatment does not inactivate MMTV or release virus from the cell. (A) Pseudovirions were incubated for 15 min at 37 °C at the indicated pH, neutralized and then used to infect mTfR1/293T cells. (B) Low pH does not release virus from the cell surface. Pseudoviruses were incubated with mTfR1/293T cells for 1 h at 4 °C, pulsed at the indicated pH and then infection at 37 °C was allowed to proceed. Colonies were counted 48 h later.

formation; hormone-stimulated cells that expressed the same levels of Env but incubated at pH 7 demonstrated no syncytia (Fig. 1A).

To determine which pH induced maximum cell–cell fusion, we incubated dexamethasone-treated cells in media ranging from pH 4.0 to 7.4. Maximal syncytia formation occurred at around pH 5.0 when either the percentage of cells in syncytia or the number of nuclei in a syncytia was measured; outside the pH 4.8 to 5.5 range, no fusion occurred (Figs. 1B and C and not shown). These data indicated that virus–cell fusion most likely occurred in late endosomes or lysosomes.

Low pH does not irreversibly inactivate MMTV

The Env proteins of viruses that require low pH to induce a conformational change are often inactivated by low pH treatment (termed premature triggering). To determine whether this was true for MMTV, we created MMTV Env-pseudotyped MLV particles, as previously described (Zhang et al., 2003) and incubated them at pH 5 for 15 min at 37 °C. The pseudoviruses were then used to infect 293T cells stably expressing mTfR1 (mTfR1/293T) (Zhang et al., 2003). Low pH (pH 5) pre-treatment had no effect on the infection levels (Fig. 2A), indicating that the MMTV Env does not undergo an irreversible change in the absence of receptor.

We also tested whether low pH treatment disrupted MMTV interaction with cells. Pseudovirions were bound to cells for 1 h at 4 °C and then treated with different pH buffers. The infection levels were the same when virus-bound cells were treated with pH 5, 6 and 7 buffer; only treatment with pH 3 buffer abolished infection (Fig. 2B). Taken together, these data indicated that low pH does not cause pre-triggering of the MMTV Env or dissociation from receptor.

MMTV traffics to both the early and late endosomes

TfR1 typically binds to holo-Tf and traffics to the early recycling endosome (~pH 6) where iron is released. The apo-Tf/receptor complex then returns to the cell surface and releases Tf. We next used immunohistochemistry to determine the intra-cellular location of MMTV. NMuMG cells (normal mouse mammary gland cells that express mTfR1) were incubated with MMTV on ice for 1 h, shifted to 37 °C for 15 min, then fixed and immunostained with anti-MMTV and anti-EEA1 or anti-rab 7 antibodies, which are markers of early and late endosomal compartments, respectively. MMTV was detected in both compartments, indicating that virus efficiently enters this pathway and consistent with the pH 5 requirement for Env-mediated cell–cell fusion (Fig. 3A). We also found that mTfR1 co-localized with a GFP-tagged version of the early endosomal marker rab5 in the presence or absence of virus, while it only was found in the same compartment as a rab7-GFP (late endosomal marker) in the presence of virus (not shown). This suggests that MMTV can re-direct trafficking of mTfR1 from the early to late endosome.

MMTV infection requires trafficking to a low pH compartment

Next, to determine whether MMTV infection required only low pH and receptor or if trafficking to an intra-cellular low pH compartment was necessary, we tested if virus could enter cells at the plasma membrane by treatment with low pH. We treated mTfR1/293T cells with either nocodazole or bafilomycin A, both of which inhibit MMTV infection (Fig. 4A and (Ross et al., 2002), incubated them with MMTV at 4 °C, pulsed them with pH 5 or pH 7 buffer and then tested for infection 2 days later by quantifying the level of MMTV DNA using real-time quantitative PCR (RT-qPCR). Treatment with either drug dramatically reduced the level of infection compared to cells that underwent the same infection protocol in the absence of drug (Fig. 4B). We also tested whether MMTV required two-step activation of fusion at the plasma membrane as is needed for ASLV (Barnard et al., 2006; Delos et al., 2005; Matsuyama et al., 2004). MMTV was incubated with mTfR1/293T cells at 37 °C for 1 h in the presence of bafilomycin A, the media was removed and then the cells were treated with pH 7 or pH 5 buffer for 15 min at 37 °C (Fig. 4C). Again, low pH buffer was unable to rescue MMTV infection at the cell surface, supporting the hypothesis that MMTV requires endosomal trafficking as well as low pH to achieve infection.

We next tested several molecules previously shown to disrupt clathrin-mediated endocytosis of TfR1 for their effects on MMTV infection. This included a dominant-negative Eps15 construct (Benmerah et al., 1999) and a molecule termed Transferrin receptor Trafficking Protein (TTP; SH3BP4), over-expression of which has been show to inhibit TfR internalization (Tosoni et al., 2005). mTfR1/293T cells were transiently transfected with GFP-tagged versions of the dnEps15 and TTP molecules and 24 h later, infected with MMTV and

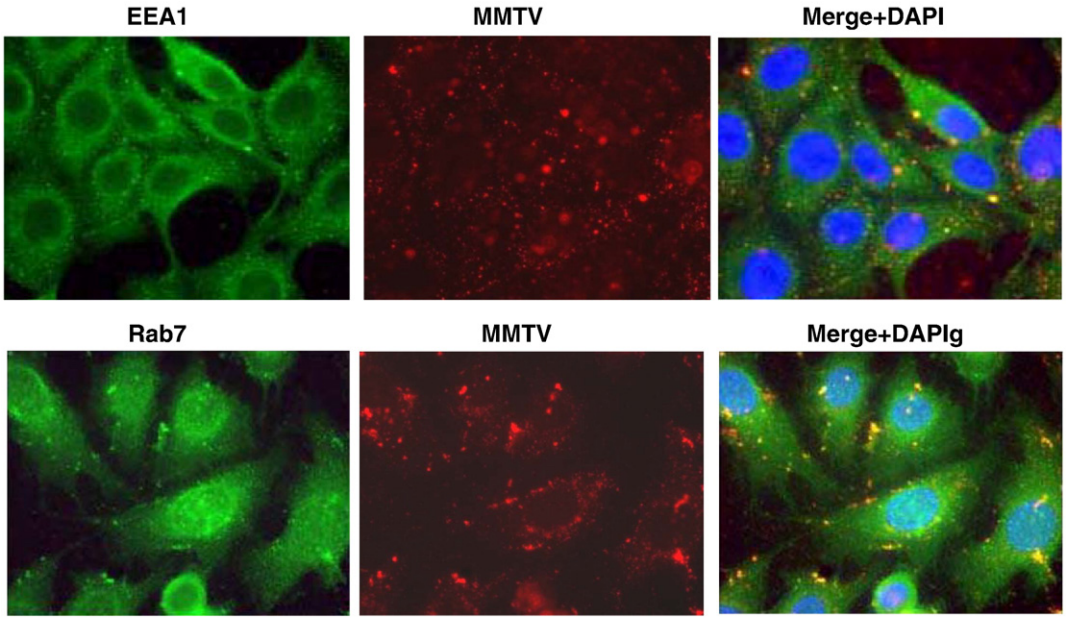


Fig. 3. MMTV localizes to the early and late endosomes. NMuMG cells were incubated with MMTV (500 particles/cell) at 4 °C for 90 min. Cells were then shifted to 37 °C for 15 min and immunostained with anti-EEA1 or with anti-rab7 antibodies. MMTV was detected by a monoclonal antibody against the MMTV capsid protein (Case et al., 2005).

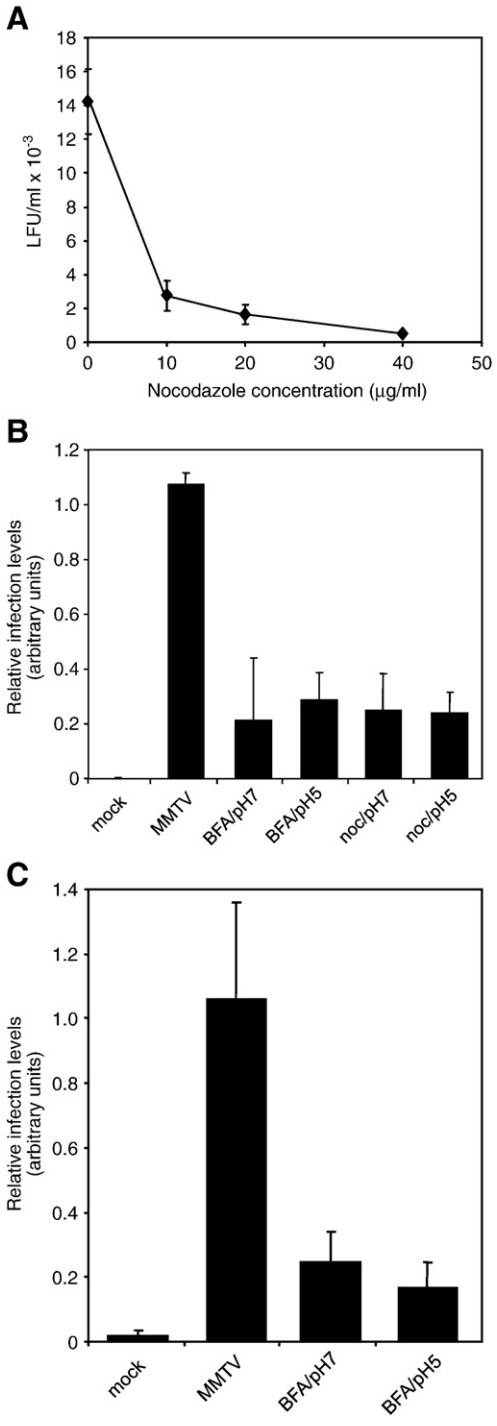


Fig. 4. MMTV requires trafficking to a low pH compartment to infect cells. (A) mTfR1/293T cells were treated with nocodazole at the indicated concentrations for 1 h and then infected with MMTV pseudovirions in the presence of drug. (B) mTfR1/293T cells were pretreated with either bafilomycin A (BFA) or nocodazole (noc) at 37 °C for 1 h. The cells were then incubated with MMTV at 4 °C for 1 h, followed by treatment with different pH buffers in presence of BFA or nocodazole at 37 °C for 15 min. The cells were washed, incubated in media containing BFA or nocodazole for two more hours and then refed. Forty-eight hours later, DNA was isolated and subjected to RT-qPCR. (C) mTfR1/293T cells were pretreated with BFA at 37 °C for 1 h, then incubated with MMTV at 37 °C for 1 h, followed by treatment with different pH buffers in presence of BFA at 37 °C for 15 min. The cells were washed, incubated in media containing BFA for two more hours and then refed. Forty-eight hours later, DNA was isolated and subjected to RT-qPCR.

VSV pseudotypes. As has been reported previously, VSV infection was greatly inhibited by the dnEps15 construct (Sun et al., 2005); over-expression of TTP also inhibited VSV infection (Fig. 5). In contrast, both molecules had only modest effects on MMTV infection (Fig. 5).

Taken together, these data indicate that MMTV cannot infect cells through the plasma membrane, but instead require trafficking to a low pH compartment. Moreover, this trafficking appears to be independent of clathrin-mediated endocytosis.

MMTV infection does not depend on protease cleavage

Recently, the entry proteins of several pH-dependent viruses have been shown to require cleavage by cathepsin cysteine proteases located in the endosomal compartment to mediate membrane fusion. We therefore tested several protease inhibitors for their effects on MMTV pseudovirus infection: E64d (cysteine proteases), leupeptin (endosomal trypsin-like serine and cysteine proteases), CA-074 (cathepsin B), Z-FY(*t*-Bu)-dmk (cathepsin L), aprotinin (serine-type proteases) and pepstatin (aspartate proteases). VSV G pseudotypes served as a negative control, Ebola virus G*P* pseudotypes as a positive control. None of these inhibitors significantly affected MMTV infection of either NMuMG (Fig. 6A) or mTfR1/293T (Figs. 6B and C) cells at the highest concentrations tested. For example, 150 μM E64d inhibited neither MMTV nor VSV pseudovirus infection (Figs. 6A and C), while this concentration completely inhibited infection by Ebola virus G protein pseudotypes (Fig. 6B); higher concentrations of E64d were toxic to cells. Thus, the MMTV Env does not require cleavage by proteases in a low pH compartment, at least in the two cell types examined here.

MMTV Env causes fusion of human cells

Previous studies with enveloped viruses that enter from low pH compartments have identified different requirements for entry. For example, the fusion activity of the influenza HA can be triggered by pH alone and does not depend on the presence of specific cell surface proteins (Skehel and Wiley, 2000). For other viruses, like avian sarcoma/leukemia virus (ASLV), receptor binding is needed to prime the viral Env for subsequent low pH treatment (Mothes et al., 2000; Delos et al., 2005). We next used Env-mediated syncytia formation to determine if fusion of Env-mediated cell–cell fusion was TfR1- as well as pH-dependent. We co-transfected 293T cells with expression vectors containing the MMTV Env together with wild type hTfR1 or a chimeric molecule, hTfR1-MTM52. MTM52 has a hTfR1 backbone, but contains amino acids 285–296 and seven amino acid substitutions

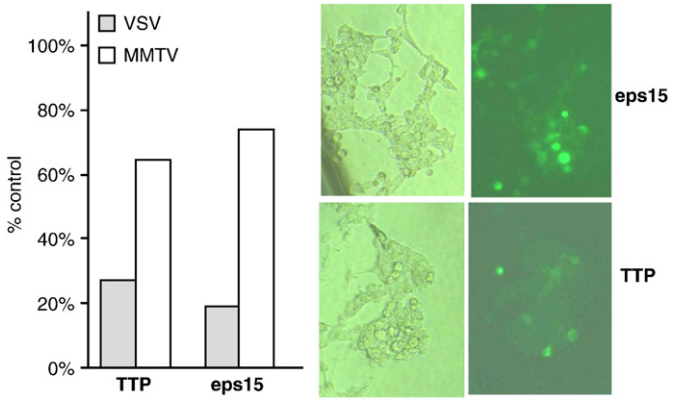


Fig. 5. MMTV infects cells in the presence of a dominant-negative Eps15 molecule and in cells over-expressing TTP. mTfR1/293T cells were transfected with the GFP-tagged Eps15 or TTP molecule. In both cases, >50% of cells were transfected (right panel). Twenty-four hours after transfection, the transfected cells, as well as untransfected controls, were infected with MMTV Env- or VSV G-pseudovirions. Colonies were counted 48 h later. Data is presented as the percent infection relative to untransfected cells. Shown is the average of 2 wells; this experiment was repeated 3 times with similar results.

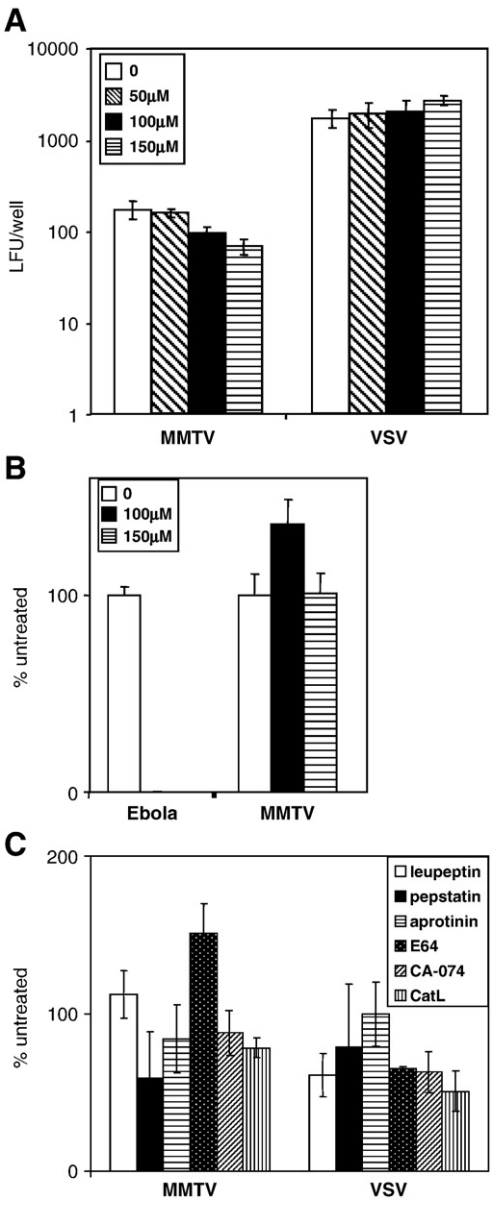


Fig. 6. MMTV does not require cathepsin cleavage to infect cells. (A) NMuMG cells grown in 24 well microtiter dishes were incubated with the indicated concentrations of E64d 2 h prior to infection with MMTV or VSV pseudovirions. Shown is the average LFU of 4 wells for each concentration of drug. This experiment was repeated 3 times with similar results. (B) mTfR1/293T cells grown in 24 well microtiter dishes were incubated with the indicated concentrations of E64d 2 h prior to infection with MMTV or Ebola pseudovirions. Shown is the average LFU of 3 wells for each concentration of drug normalized to the infection level for untreated cells in the same experiment. (C) mTfR1/293T cells were treated with 59 μM leupeptin, 4 μM aprotinin, 36 μM pepstatin, 150 μM E64d, 100 μM CA-074 or 25 μM Z-FY(*t*-Bu)-dmk for 1 h prior to and during infection with MMTV Env- or VSV G-pseudotyped MLV virus. Shown is the average LFU of 3 to 4 wells for each concentration of drug normalized to the infection level for untreated cells in the same experiment.

in the region from amino acids 569–589 from mTfR1. Introduction of these changes into the extra-cellular domain of hTfR1 renders it capable of serving as an MMTV entry receptor (Wang et al., 2006). Surprisingly, the MMTV Env induced syncytia formation to similar extents in cells expressing either hTfR1 or MTM52; indeed, Env also caused 293T cells expressing only endogenous levels of TfR1 to fuse after pH 5 treatment (Fig. 7A). Moreover, the MMTV Env induced syncytia on D17 dog and CFRK cat cells after pH 5 treatment (not shown); neither the dog nor the cat TfR1 support MMTV pseudovirus infection (Wang et al., 2006).

To determine whether Env-mediated syncytia induction of human cells relied on hTfR1, we grew Env-expressing 293T cells in high iron, an established method for decreasing receptor expression (Casey et al., 1989). Total and surface hTfR1 expression levels, as determined by Western blot and FACS, respectively, were diminished by at least 10-fold by this treatment, without affecting Env expression (Fig. 7B). High iron treatment also abolished syncytia induction (Fig. 7C). To control for non-specific effects of high iron on syncytia formation, we also introduced a Moloney murine leukemia virus Env that induces cell fusion at neutral pH into 427 cells [293T cells expressing MCAT-1, the MoMLV receptor (Chung et al., 1999)], and showed that it induced syncytia to a similar extent in the presence and absence of iron (Fig. 7C).

We also tested whether MMTV Env required binding to receptor in addition to low pH to induce syncytia. We transfected an expression plasmid encoding an Env with point mutation in the receptor binding site that abrogates MMTV pseudovirus binding and infectivity (Zhang et al., 2003) into 293T cells and showed that low pH/Env-dependent cell–cell fusion was completely abolished (Fig. 7D). Thus, low pH alone was not sufficient to induce the conformational change in Env; receptor binding was also required to achieve virus–cell membrane fusion.

MMTV Env binds hTfR1

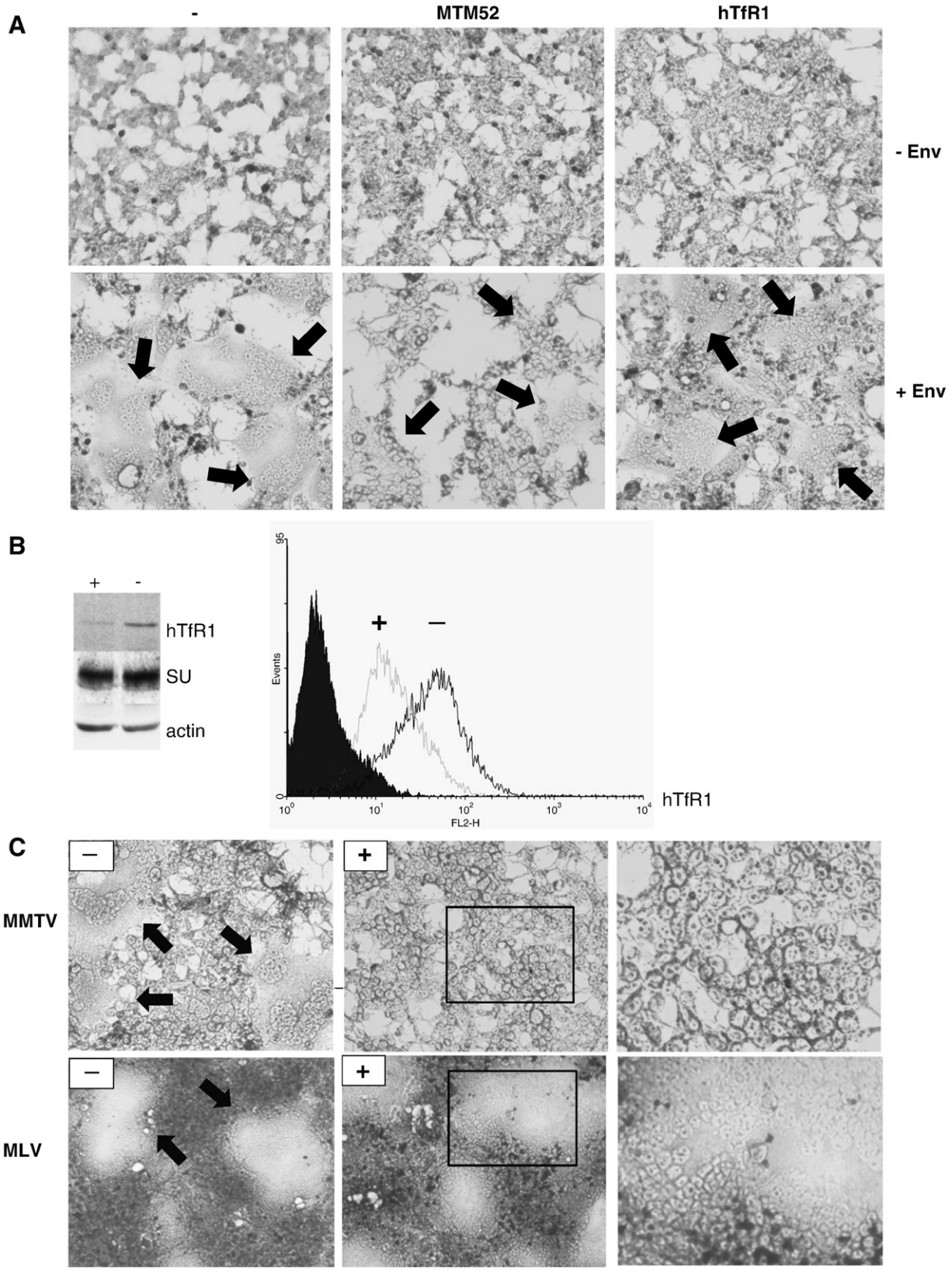
We previously determined that two segments of mTfR1, physically disparate from the Tf binding site but next to each other on the crystal structure, were necessary to convert the hTfR1 into an MMTV entry receptor (Wang et al., 2006). Additionally, sequence comparison and mutagenesis studies showed that all functional MMTV entry receptors (mouse and rat) have a similar pattern of charged amino acids in the binding site, compared to nonfunctional receptors (human, cat, dog). However, the cell–cell fusion data presented in the preceding section indicated that MMTV might bind hTfR1. To determine if this was the case, we performed co-transfection/co-immunoprecipitation studies with expression plasmids encoding the MMTV Env, hTfR1 or MTM52 (Wang et al., 2006). When anti-MMTV Env antiserum was used for the immunoprecipitation step and anti-hTfR1 antibodies for the detection step, equal levels of both MTM52 and hTfR1 were precipitated, even under high stringency conditions (0.5 M KI) (Fig. 8). Thus, at least in an over-expression assay, both the hTfR1 and mTfR1 bind to the MMTV Env.

MMTV internalization occurs in cells expressing mTfR1 but not hTfR1

Although the MMTV Env bound hTfR1 and mediated cell fusion in the presence of hTfR1, we have been unable to demonstrate that the human receptor supports MMTV infection (Ross et al., 2002; Wang et al., 2006; Zhang et al., 2003). Our data thus far indicated that during infection, MMTV virions bound to mTfR1 on the cell surface are endocytosed and traffic to the late endosome or lysosome to reach the correct pH compartment for entry. Previously, we showed that MMTV binding to the surface of mouse cells resulted in decreased surface expression of TfR1, indicating that the virus triggered receptor internalization (Ross et al., 2002). To determine if MMTV induced internalization of the hTfR1, 293T or mTfR1/293T cells were spinoculated for 2 h at RT with MMTV virions, immunostained with anti-human or anti-mouse TfR1 antibodies and analyzed by FACS. MMTV infection down-regulated the levels of mTfR1 on mTfR1/293T cells, but did not affect hTfR1 levels on human 293T cells (Fig. 9A).

As a second assay to determine whether the hTfR1 supported MMTV internalization, we used MMTV Env pseudotypes that incorporated a MLV matrix (MA) protein tagged with GFP; similar constructs have been used to follow HIV-1 entry in tissue culture cells

using fluorescence microscopy (Hubner et al., 2007; Muller et al., 2004). The GFP-tagged pseudovirions were incubated with either 293T or mTfR1/293T cells on ice for 1 h and then shifted to 37 °C for different times. After incubation for 5 min most of the virus was on the surface of both the mTfR1/293T and 293T cells (Fig. 9B, panels a and c, respectively). After 30 min, the majority of virus bound to mTfR1/293T cells was internalized (Fig. 9B, panel b). In contrast, the virus on 293T cells largely remained on the cell surface (Fig. 9B, panel d). Together, these data indicated that MMTV binding to hTfR1 did not trigger receptor endocytosis.



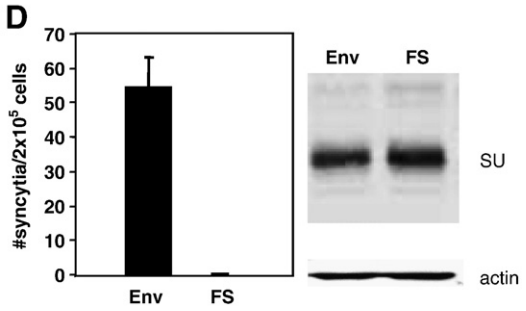


Fig. 7. The MMTV Env induces receptor-dependent fusion of human cells. (A) Human 293T were transiently transfected with the hTfR1 and the infection-competent mouse/human hybrid MTM52 expression plasmids in the presence and absence of an Env expression plasmid. Forty-eight hours post-transfection, the cells were treated with pH 5.0 media. Representative syncytia are marked by arrows. (B) 293T cells were grown in the presence (−) or absence (+) of iron for 24 h, transiently transfected with the MMTV Env expression plasmid and then grown for an additional day +/− iron. MMTV Env and hTfR1 expression were analyzed by Western blotting. hTfR1 cell surface expression was analyzed by FACS using PE-conjugated anti-hTfR1 antibody; filled curve, unstained cells; grey line, cells treated with iron; black line, untreated cells. (C) Env-transfected 293T cells, as described in A were grown in the absence (−) and presence (+) of iron. 293T cells were transfected with MMTV Env expression vector and treated with pH 5.0 media to induce syncytia or 427 cells transfected with MoMLV Q123 expression vector were grown to confluence. The enlarged area within the box is shown in the far right panels. (D) Syncytia induction requires a receptor binding-competent Env. 293T cells were transiently transfected with expression vectors encoding wild type Env or Env-FS, which has a point mutation in the receptor binding site (Zhang et al., 2003). Low pH treatment was performed 48 h after transfection Env and the number of syncytia/20,000 cells was counted. Inset: Western blot analysis with anti-MMTV antiserum was

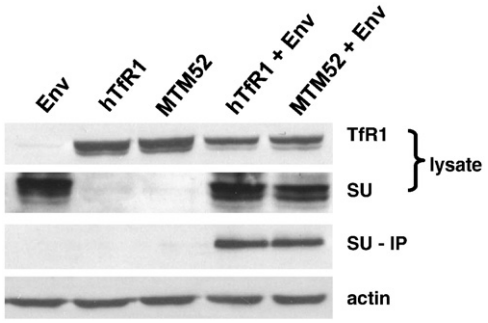


Fig. 8. MMTV SU binds both mTfR1 and hTfR1. (A) Human 293T cells were transfected with Env, hTfR1 or MTM52 alone, or Env was co-transfected with hTfR1 and MTM52. Immunoprecipitations of cell lysates were performed with goat anti-MMTV antiserum and Western blots were probed with the anti-hTfR1 monoclonal antibody OKT-9 (IP); cell lysates were also analyzed by Western blots to examine the level of TfR1 expression (lysates). The co-immunoprecipitations were performed at 4 °C in the presence of 0.5 M KI.

Finally, we tested whether low pH treatment of hTfR1-expressing cells would allow infection by MMTV at the cell surface. 293T were transfected with hTfR1 or MTM52 expression vectors and then incubated with MMTV at 4 °C to allow binding. The cells were then treated with pH 5, pH 6 or pH 7 media for 15 min at 37 °C and virus was removed by washing; 48 h later, infection was analyzed by RT-qPCR, using primers specific for the viral DNA. Although MMTV infected MTM52-expressing cells under all conditions, we were unable to detect infection of hTfR1-transfected 293T cells, even though they expressed increased levels of hTfR1 compared to untransfected cells (Fig. 10). Taken together, these results showed that although MMTV binds human TfR1, it is only endocytosed when bound to the infection-competent mouse receptor and thus cannot use the human receptor for infection.

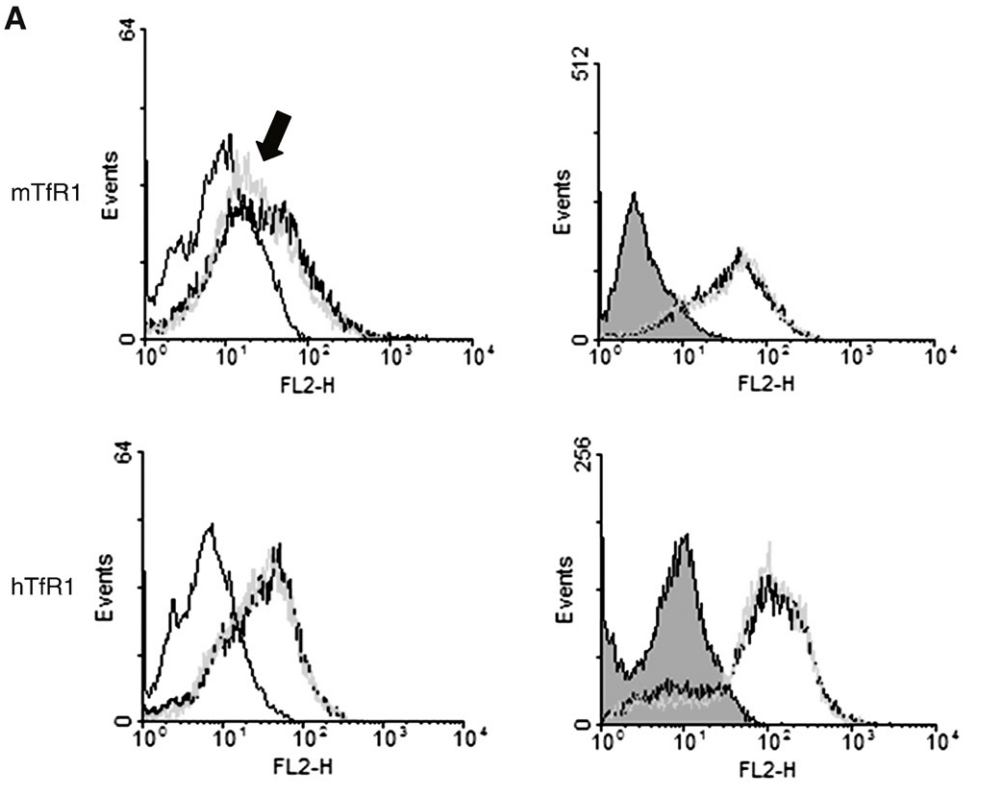
Discussion

The molecules that viruses bind on the cell surface constitute a diverse collection of cellular proteins, carbohydrates, and lipids. These receptors differ from one virus to the next and their expression ranges from abundant and ubiquitous to limited and restricted. Some molecules merely serve as attachment factors that concentrate viruses on the cell's surface. Particularly for enveloped viruses, many receptors not only bind viruses but induce conformational changes in the virus attachment proteins that are required for virus entry. Other receptors also guide bound viruses into endocytic pathways and may transmit signals to the cytoplasm required for entry (Smith and Helenius, 2004).

Our data suggest that mouse TfR1 provides all three functions for MMTV, virus attachment, induction of the Env conformational change and trafficking to a low pH compartment. Typically, receptors follow three potential routes after exiting the sorting endosome, trafficking either back to the cell surface via the recycling endosome, to the trans-Golgi network or, if targeted for degradation to the late endosome/lysosome (≤pH 5.5) (Gruenberg and Stenmark, 2004). In its normal metabolic role, TfR1 traffics first to the sorting endosome (~pH 6) upon binding diferric Tf and then moves either directly or via the recycling endosome (also ~pH 6) back to the surface where apo-Tf is released. Given that MMTV Env-mediated membrane fusion occurs at pH 5 but not pH 6 and its association with the late endosome (Fig. 3A), it is likely that binding of virus re-targets TfR1 to this compartment. Indeed, our preliminary studies indicate that this is the case (not shown). MMTV may re-direct TfR1 to the late endosome to gain better access to the nucleus, since the sorting endosome is believed to be located in the cell periphery, while the late and recycling endosomes are found in the perinuclear region.

TfR1 endocytosis via clathrin-coated pits has been shown to require a number of molecules, including TTP and Eps15. TTP is a newly identified molecule which has been shown to be important for internalization of TfR1, probably through its interaction with dynamin (Tosoni et al., 2005). We have also found that over-expression of a dominant-negative form of dynamin had little or no effect on MMTV infection (not shown). Eps15, which is associated with the adaptor protein AP-2, has been implicated in the endocytosis and entry of a number of enveloped viruses, including Sindbis virus and VSV (Carbone et al., 1997; Sun et al., 2005). However, MMTV infection occurred at almost wild type levels in cells expressing the dominant-negative Eps15, indicating that its trafficking to the late endosome is largely independent of the normal endocytic pathway taken by TfR1. Several other viruses, notably severe acute respiratory syndrome (SARS) and mouse hepatitis virus 2 (MHV2), which like MMTV require a <pH 5.5 compartment for entry, have recently been shown to infect cells in the presence of the dominant-negative Eps15 (Pu and Zhang, 2008; Wang et al., 2008). Interestingly, it has been previously suggested that intra-cellular trafficking of TfR1 depends not only on targeting sequences found in the receptor but also on the particular ligand bound to the receptor, since a genetically engineered Tf that bound to a different region of the receptor redirected it to the lysosome and targeted it for degradation (Zaliauskiene et al., 2002). Since MMTV interacts with a region of TfR1 distinct from Tf, it is possible that the virus also re-directs the receptor from the early recycling endosome to a more acidic compartment.

Fig. 9. Virus and receptor are internalized in cells expressing mouse but not the human TfR1. (A) 293T and mTfR1/293T cells were spinoculated with MMTV virions at room temperature for 2 h and then immunostained with PE-conjugated anti-human (293T) or anti-mouse (mTfR1/293T) TfR1 antibodies and subjected to FACS analysis. Dotted line, unstained cells; light grey line, cells incubated with virus; solid black line, cells spinoculated in the absence of virus. (B) MMTV pseudovirions containing a GFP-tagged MLV matrix protein were incubated with either mTfR1/293T (a and b) or 293T (c and d) cells grown on glass slides on ice for 1 h. The cells were moved to 37 °C for 5 min (a and c) or 30 min (b and d). Cells were fixed with paraformaldehyde and stained with rhodamine-conjugated phalloidin and examined by fluorescence microscopy. Magnification: 630×.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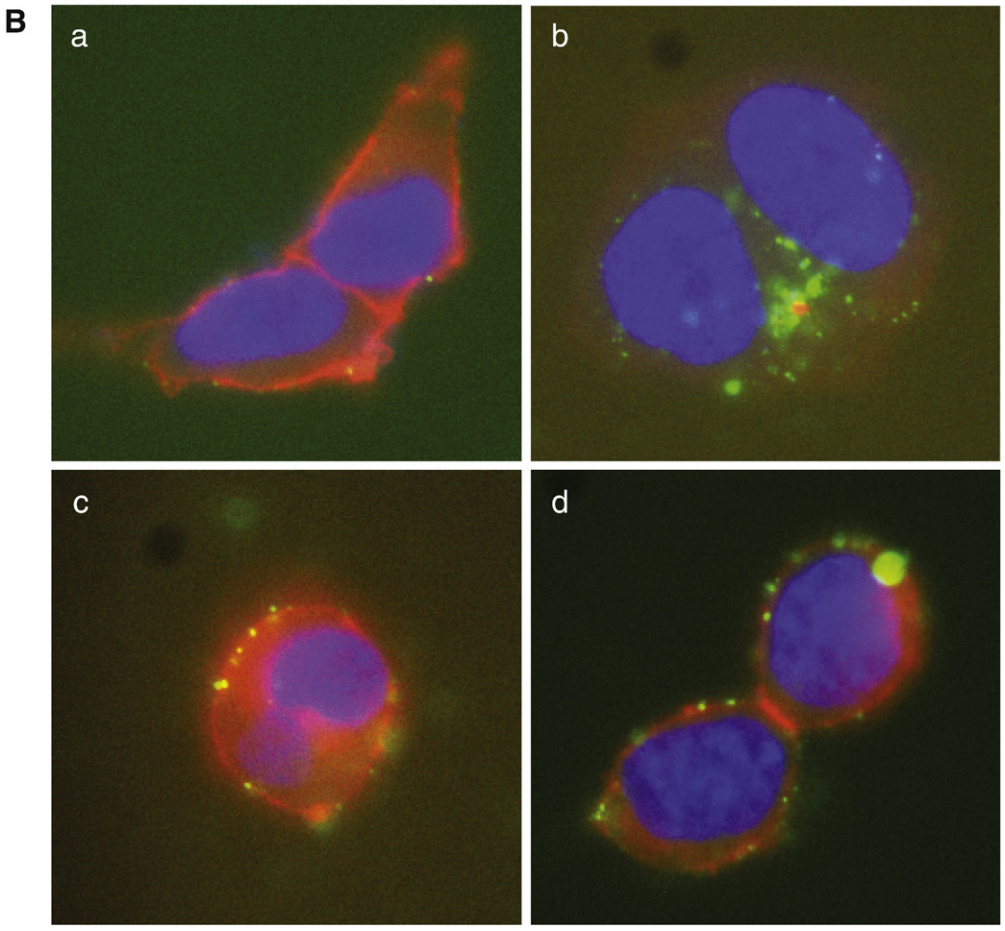
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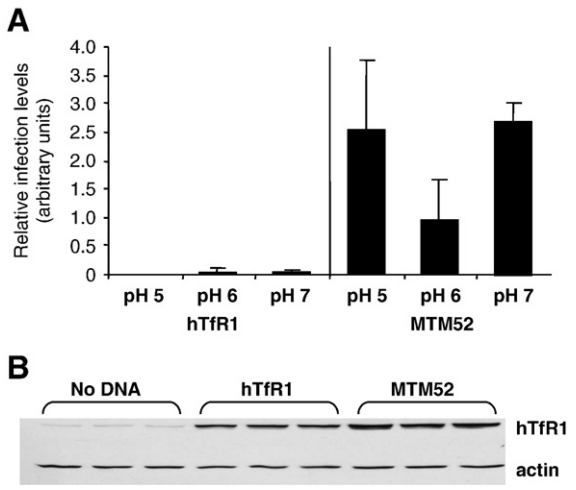

Fig. 10. Low pH is not sufficient to achieve MMTV infection of human cells. (A) 293T were transfected with hTfR1 or MTM52, incubated with MMTV on ice, treated with different pH buffers at 37 °C and then refed. After 48 h, DNA was extracted and RT-qPCR was performed with primers that amplify a region in the MMTV LTR; the MMTV signals were normalized to GAPDH. (B) Western blot analysis of transfected cells.

We show here that unlike many other enveloped viruses, while binding to receptor and low pH are necessary, they are not sufficient for MMTV infection. Infection by several viruses, including Ebola, MLV, Hendra and the SARS and MHV coronaviruses have been shown to require cleavage by cysteine proteases located in the late endosome/lysosome, such as cathepsin B and L, to achieve efficient infection (Chandran et al., 2005; Kaletsky et al., 2007; Kumar et al., 2007; Pager and Dutch, 2005; Qiu et al., 2006; Schornberg et al., 2006; Simmons et al., 2005). However, we found no evidence that MMTV required cleavage by such enzymes, since neither specific inhibitors of cathepsin (E46d) or more general protease inhibitors (leupeptin, aprotinin, etc.) affected MMTV infection levels. Kumar et al. have also suggested that MLV might require cathepsins at a post-membrane fusion stage of infection, since they found that VSV G/MLV pseudotype infection of NIH3T3 cells was inhibited by cathepsin inhibitor III and CA-074 (Kumar et al., 2007). In our studies, VSV G/MLV pseudotype infection was not affected by any of the cathepsin inhibitors; this difference could be due to our use of NMuMG (mouse mammary epithelial) and 293T (human kidney epithelial) cells as opposed to NIH3T3 cells used in the previous study.

Our results also argue that MMTV cannot infect human cells using the endogenous TfR1. Previously we showed that MMTV used mouse but not human TfR1 as its entry receptor and that the regions of receptor required for infection mapped to two segments that formed a potential binding site consisting of a unique charged amino acid-distribution pattern (Wang et al., 2006). However, here we show that MMTV binds both human and mouse TfR1 (Fig. 8) and that the Env protein mediates cell–cell fusion in its presence at low pH (Fig. 7). However, neither infectious virus particles nor pseudovirions were capable of triggering receptor internalization (Fig. 9). Although the MMTV Env binds hTfR1 even under high stringency conditions, it is possible that binding is weaker than to the mouse receptor. Thus, under the conditions of high Env expression achieved after transfection, there would be a sufficient number of Env molecules that undergo the conformational change required to induce Env-mediated cell–cell fusion. Alternatively, MMTV may bind to a site on hTfR1 that does not trigger receptor internalization via the YTRF motif in the cytoplasmic tail; the YTRF motif has been shown to be important for Tf-mediated TfR1 endocytosis via clathrin-coated pits (Collawn et al., 1993).

It is not surprising that low pH alone does not trigger an irreversible conformational change in the MMTV Env, since the virus is acquired through milk and must survive the low pH environment of the digestive tract. Importantly, we show here that to achieve entry, MMTV must direct the TfR1 not to its normal recycling compartment, but to a lower pH compartment such as the late endosome/lysosome. Why does MMTV require trafficking with TfR1 to a pH 5 compartment? Clearly, neither receptor binding nor low pH is sufficient, since virus did not enter cells that express elevated levels of human or mouse TfR1 at the cell surface even when incubated at pH 5. Marsh and Bron have previously reported that fusion at the cell surface does not allow Semliki Forest virus (SFV) infection and suggested that the trafficking to an endocytic compartment could provide a means for bringing viral particles to sites in the cell where replication can proceed (Marsh and Bron, 1997). It is also possible there are late endosomal co-factors or co-receptors other than cathepsins that are necessary for infection by MMTV and other viruses, a possibility which is currently under investigation.

Materials and methods

Cell lines and plasmids

293T human kidney epithelial were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin (50 μg/ml). mTfR1/293T (TRH3 in (Zhang et al., 2003) and 427 (293T cells stably expressing murine leukemia virus receptor) (Chung et al., 1999) cells were grown in the same medium supplemented with geneticin (100 μg/ml) and NMuMG (normal murine mammary gland) and Mm5MT cells in the same media containing insulin (10 μg/ml). Plasmids pEnv and pEnv-FS containing wild type MMTV *env* and an *env* with a mutation in the receptor binding site, respectively, were previously described (Zhang et al., 2003). Plasmid Q123 contains a variant MLV Env lacking the R peptide that induces cell–cell fusion at neutral pH (Zavorotinskaya et al., 2004). The GFP-tagged TTP, Eps15, rab5 and rab7 and hTfR1 and MTM52 (an engineered human TfR1 that supports MMTV entry and can be detected by the anti-hTfR1 monoclonal antibody OKT-9) constructs were previously described (Tosoni et al., 2005; Benmerah et al., 1999; Meertens et al., 2006; Wang et al., 2006).

Co-immunoprecipitations

Cells were transfected via the calcium phosphate method. Briefly, 2×10^6 293T cells per well were seeded the day before transfection. Twenty-four hours later, the cells were refed with medium containing 10 ng/ml sodium butyrate to induce expression of the CMV promoter-driven genes. Twenty-four hours later, cell lysates were prepared by incubating cells in 0.5 M KI co-immunoprecipitation buffer (20 mM Tris–Cl, pH 7.5, 0.5 M KI, 1% NP-40, 1 mM EDTA, 1 μg/ml Protease inhibitor cocktail, Sigma at St. Louis, MO). Goat anti-MMTV antisera was added to the lysates and incubated overnight at 4 °C. The immune complexes were precipitated with Protein G agarose (Invitrogen, Carlsbad, CA) and proteins eluted in SDS-protein loading buffer. The proteins were resolved by SDS-polyacrylamide gels (SDS-PAGE) and analyzed by Western blotting, as previously described (Zhang et al., 2003). Hybridoma supernatant OKT-9 (a gift from Morrie Birnbaum) was used to detect hTfR1 and MTM52 and monoclonal anti-SU to detect MMTV. For whole cell lysates, the blots were stripped and probed with anti-human beta-actin (Santa Cruz Biotechnology, Inc.).

Virus entry assays

GFP-labeled MMTV Env-pseudotyped MLV recombinant viruses were produced by transient co-transfection of 293T cells with plasmid pENV, pHit111 (murine leukemia virus [MLV] genome with the β-galactosidase marker), pHit60 (MLV *gag/pol* genes) (Soneoka et al., 1995), and Matrix-GFP (the MLV matrix protein with an in-frame fusion to E-GFP) as previously described (Golovkina et al., 1998). The

supernatant was harvested at 48 h post-transfection and concentrated by ultracentrifugation, 25,000 rpm for 2 h in an SW28 rotor at 4 °C. Virus titers were measured by counting β-galactosidase-positive colonies (reported as LacZ-forming units (LFU) or fluorescence microscopy to detect GFP-positive colonies. For examining virus entry, the GFP-labeled virus was incubated with either 293T cells or mTfR1/293T cells in the presence of 8 μg/ml polybrene at 4C for 1 h. The cells were then shifted to 37 °C for 5 or 30 min, as indicated in the figure legend, washed with cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and stained with rhodamine phalloidin (Molecular Probes, Inc., Eugene, Oregon). Cells were visualized by fluorescence microscopy (Axioplan 2 Imaging, ZEISS, Inc., Denver, CO).

Immunohistochemistry

NMuMG cells seeded on collagen Type I Cellware 8-well Culture-Slide (BD Biosciences, Bedford, MA, USA) were incubated with virus on ice for 1 h, then shifted to 37 °C for 15 min. The cells were fixed with 3% paraformaldehyde in PBS (pH 7.4) for 15 min, quenched with 50 mM NH_4Cl, and permeabilized with 0.1% Triton X-100. After blocking with 10% goat serum, the cells were incubated with primary and secondary antibodies for 30 min each and mounted DAPI-containing mounting medium (Vector Laboratories, Inc. Burlingame, CA). The MMTV capsid protein was detected using a monoclonal antibody kindly provided by Dr. Tatyana V. Golovkina. Early and late endosomes were detected by goat anti-mouse polyclonal IgGs against early endosome autoantigen 1 (EEA1) and Rab7 (Santa Cruz Biotechnology, INC. Santa Cruz, CA, USA), respectively. Secondary antibodies used were Alexa Fluor 568 (red) goat anti-mouse IgG (Molecular Probes, Eugene, Oregon, USA) or fluorescein (FITC)-conjugated affinity pure donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, INC. West Grove, PA). Cells were viewed with a 63× objective lens and images were captured with Slidebook 3.0 software (Leeds Precision Instruments, Inc. Minneapolis, MN) before being transferred into Adobe Photoshop.

Receptor internalization assay

Virions were obtained from the NCI repository and stored at -70 °C. Infection of 293T and mTfR1/293T cells was performed with 500 virus particles/cell by spinoculation in the presence of 8 μg/ml polybrene, as previously described (Zhang et al., 2003) at either room temperature or 4 °C. Cells were stained with phycoerythrin (PE)-conjugated anti-human CD71 (BD Pharmingen, Inc., location) or anti-mouse CD71 (BD Pharmingen, Inc.) to detect TfR1 levels on 293T or mTfR1/293T cells, respectively and analyzed by FACS analysis as described above.

Cell–cell fusion assays

Mm5MT cells were grown in 0.5 μM dexamethasone 24 h prior to fusion induction to increase cell surface Env expression. 293T, CRFK and D17 cells were transfected with MMTV Env, hTfR1, MTM52 or Env+hTfR1, Env+MTM52 plasmids and 48 h post-transfection, cells were treated with citrate buffer at the pH indicated in the figure legend for 15 min to induce fusion. The cells were refed with growth medium and incubated at 37 °C overnight, washed with PBS and stained with Giemsa-Wright. The number of syncytia in each well was counted and number of syncytia/number of total cells was used to quantitate the cell–cell fusion.

Infection assays

293T cells were transfected with a mTfR1 expression plasmid, as described above. Twenty-four hours post-transfection, diluted pseudovirus supernatants containing polybrene (8 μg/ml) were incubated with cells at 37 °C for 2 h. The cells were stained for β-galactosidase activity 48 h after infection, and blue colonies were counted. Data are presented as LacZ-forming units (LFU) per ml of supernatant. For the protease inhibitor experiments, NMuMG or mTfR1/293T cells were infected with MMTV, VSV or Ebola pseudoviruses. E64d, leupeptin, CA-074, Z-FY(*t*-Bu)-dmk, aprotinin and pepstatin were added to the infection media at the indicated concentrations for 2 h prior to and during the 2 h incubation with virus. The cells were washed and stained for β-galactosidase activity 48 h post-infection.

Low pH infection assay

293T and mTfR1/293T cells were incubated with virus (500 virus particles/cell) at 4 °C or 37 °C for 1 h. The cells were washed with cold PBS and then different pH (pH 5, 6, 7) citrate buffer (40 mM sodium citrate, 10 mM KCl, 135 mM NaCl) were used to treat the cells for 15 min at 37 °C. Fresh media was added to the cells. Forty-eight hours post-infection, DNA was extracted with DNAEasy kit (Qiagen, Inc., Maryland) and subjected to real-time quantitative PCR (RT-qPCR). MMTV primers that amplify the MMTV(C3H) LTR were used to detect infection, as previously described (Courreges et al., 2007). Primers to GAPDH were used to normalize input DNA.

Receptor down-regulation

Duplicate cultures of 293T and 427 cells were incubated with ferric ammonium citrate (100 μg/ml) (FeAC) for 24 h prior to transfection. The cells were washed and transfected with plasmids pENV or Q123. Twelve hours post-transfection, the cells were refed with media containing FeAC. Twenty-four hours later, one culture was used for cell–cell fusion assay, as described above. A second culture was used to determine total TfR1 levels by Western blotting with OKT-9 or surface TfR1 levels by FACS with phycoerythrin-conjugated anti-human CD71 antibodies.

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