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Promotion of retroviral entry in the absence of envelope protein by chlorpromazine

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Abstract

Retrovirus packaging cell lines that express the Moloney murine leukemia virus gag, pol, and env genes and a retroviral vector genome can produce virus particles that are capable of transducing cells. Normally if the packaging cell line does not produce a functional viral fusion glycoprotein, such as the retroviral envelope protein or a foreign viral glycoprotein, then the viruses will be incapable of transducing cells. We have found that incubating envelope protein-deficient virus particles bound to cells with chlorpromazine leads to transduction. Chlorpromazine (CPZ) is a membrane-active reagent that is commonly used to induce the hemifusion to fusion transition when membrane fusion is mediated by partially defective viral glycoproteins. The concentration and pH dependence of the promotion of transduction by CPZ is consistent with a role for CPZ micelle formation in viral entry. These data indicate that caution is warranted when experiments concerning membrane fusion completion promoted by CPZ are analyzed.

Keywords: Retrovirus; Murine leukemia virus; Chlorpromazine; Membrane fusion; Hemifusion; Envelope protein; Viral entry; Glycoprotein; Gene transfer

Introduction

Recombinant viruses derived from the Moloney murine leukemia virus (Mo-MuLV), a gammaretrovirus, are commonly used for gene transfer and gene therapy applications. The Mo-MuLV envelope (Env) protein is also a model for understanding the structural and biochemical bases of membrane fusion and viral entry. The envelope protein precursor is proteolytically processed into two subunits (Henderson et al., 1984; Witte et al., 1977), SU and TM, which are linked through a disulfide bond (Pinter and Fleissner, 1977; Pinter et al., 1997, 1978). SU is found exclusively on the outside of the retroviral particle, whereas TM possesses extraparticle, a membrane-spanning, and intraparticle domains (Pinter and Honnen, 1983). The TM protein possesses at its amino terminus a sequence that is believed to encode the fusion peptide, which, upon exposure, promotes the first steps in the membrane fusion process that occurs during viral entry (Jones and Risser, 1993; Taylor et al., 2001; Zhu et al., 1998). The membrane-spanning domain of TM has recently been shown also to participate in the process of membrane fusion (Taylor and Sanders, 1999). A current model is that the binding of SU to the cellular receptor results in a thiol–disulfide exchange reaction leading to the elimination of the disulfide bond between the SU and TM subunits and consequent exposure of the TM fusion peptide that promotes membrane fusion and viral entry (Pinter et al., 1997; Sanders, 2000).

As part of our investigations of the biochemical and biophysical basis of Mo-MuLV Env-mediated membrane fusion, we were interested in utilizing the membrane-active tertiary amine chlorpromazine (CPZ) as a tool for understanding the biophysical transitions that occurred during the fusion process. In its neutral form CPZ is membrane permeable and can translocate from the outer leaflet of a cell membrane to the inner leaflet. Upon translocation CPZ becomes charged and remains kinetically trapped in the inner leaflet of the membrane (Cohen and Melikyan, 1998). CPZ has been utilized in studying the transition from the

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presumed hemifusion intermediate state in viral glycoprotein-mediated membrane fusion to complete membrane fusion, because it induces positive curvature in the inner leaflet of the membrane and thereby promotes fusion pore formation and ultimately the completion of the membrane fusion process (Melikyan et al., 1997).

CPZ has commonly been used in experiments concerning cell–cell fusion mediated by viral glycoproteins to gain evidence about hemifusion intermediates (Chernomordik et al., 1998; Markosyan et al., 2000; Melikyan et al., 1997, 1999). When cells have undergone hemifusion, the addition of CPZ will promote complete fusion (Cohen and Melikyan, 1998). On the other hand, if hemifusion has not occurred, the addition of CPZ will not promote fusion. We have found that recombinant Mo-MuLV particles that do not bear a viral envelope protein and are consequently incapable of transducing cells can introduce genetic material into a cell in a stable fashion when the virus and cells are incubated with CPZ. These data suggest that caution should be exercised when interpreting the effects of CPZ on viral glycoprotein-mediated membrane fusion.

Results

Addition of CPZ to NIH 3T3 cells incubated with Mo-MuLV lacking an envelope protein induces transduction

Recombinant Mo-MuLV particles carrying a retroviral vector encoding a nuclear-localized *Escherichia coli* β-galactosidase but lacking a viral envelope protein are produced by gpnlslacZ cells (Sharkey et al., 2001). These virus particles are incapable of transducing any of a number of cell lines that we have tested, including mouse fibroblast NIH 3T3 cells, which are susceptible to wild-type Mo-MuLV.

Mo-MuLV particles from gpnlslacZ cells or from gpnlslacZ cells that have been transfected with penv 1 min, which encodes the wild-type Mo-MuLV Env, were incubated with NIH 3T3 cells for 4 h. The cells were then treated with 0.5 mM CPZ for 1 min and incubated with normal medium for 48 h. The cells were stained with the β-galactosidase detection reagent X-gal 48 h after the CPZ treatment. The level of transduction was evaluated by determining the percentage of cells expressing β-galactosidase activity. It was found that CPZ did not affect the level of transduction (approximately 70% of the NIH 3T3 cells were stained) by Mo-MuLV particles bearing the Mo-MuLV Env protein. CPZ treatment did, however, promote transduction of on average 1% of the NIH 3T3 cells by the Mo-MuLV particles lacking an envelope protein (Mo-MuLV/Env). The percentage of cells that were transduced was stable as they were passaged for 2 weeks, which indicates that retroviral transduction and not pseudotransduction (Gallardo et al., 1997; Liu et al., 1996) was occurring. It should be noted that the level of CPZ-mediated transduction was consistently less than that of Mo-MuLV Env-mediated transduction. There was also some variation in the absolute level of CPZ-mediated transduction from experiment to experiment.

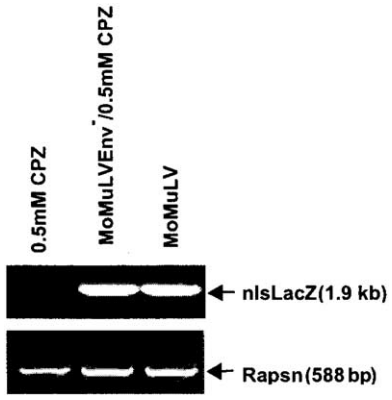

Fig. 1. Detection of retrovial vector DNA in cells transduced by Mo-MuLV lacking an envelope protein in the presence of CPZ. DNA encoding the *nlslacZ* gene amplified from cells transduced by recombinant Mo-MuLV or Mo-MuLV lacking an envelope protein (Mo-MuLV/Env$^-$) was analyzed. The *nlslacZ* gene is detected in NIH 3T3 cells incubated with Mo-MuLV/Env$^-$ and 0.5 mM CPZ but not in cells incubated with 0.5 mM CPZ alone.

To confirm that authentic transduction by Mo-MuLV particles lacking an envelope protein was occurring an enrichment of transduced cells was performed. The cells were incubated with the fluorogenic β-galactosidase substrate fluorescein di-β-D-galactopyranoside (FDG) and cells that were positive for fluorescence were sorted by flow cytometry and further passaged. The *nlslacZ* gene, amplified by the polymerase chain reaction, could be detected in the DNA from cells transduced by envelope-bearing Mo-MuLV or by Mo-MuLV/Env$^-$ in the presence of 0.5 mM CPZ but not from cells that were incubated with 0.5 mM CPZ in the absence of Mo-MuLV particles. These data indicate that the stable transduction of the cells by Mo-MuLV/Env$^-$ was promoted by CPZ incubation (Fig. 1).

Virus and CPZ concentration dependence of transduction

We wished to investigate the dependence of transduction by Mo-MuLV lacking an envelope protein upon the concentration of virus particles. As the virus is diluted, transduction decreased correspondingly (Fig. 2), indicating that there are no threshold or higher order effects of viral concentration upon CPZ-mediated transduction.

Increasing the concentration of CPZ used for treating cells incubated with virus lacking an envelope protein resulted in an increased percentage of cells transduced (Fig. 3). These data are consistent with a role for CPZ micelle formation in the promotion of transduction (Wajnberg et al., 1988). Another means of manipulating the concentration of CPZ in the membrane-permeable neutral form is by controlling the pH of the medium. Decreasing the pH decreases the concentration of the neutral form of CPZ and decreases CPZ-mediated transduction (Fig. 4).

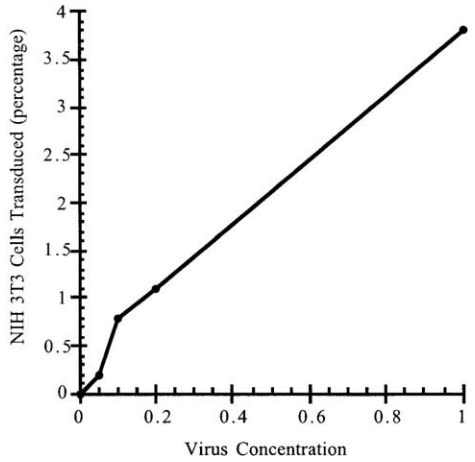

Fig. 2. Dependence of CPZ-induced transduction by Mo-MuLV lacking an envelope protein on virus concentration. Various concentrations of Mo-MuLV lacking an envelope protein were incubated with NIH 3T3 cells for 4 h prior to treatment with 0.5 mM CPZ. The level of transduction was determined 32 h after treatment by staining with X-gal. The data are representative of three independent experiments.

It is noteworthy that an important limitation on utilizing CPZ for the promotion of transduction is the cellular toxicity associated with longer incubations with or higher concentrations of CPZ (data not shown). Experiments conducted with virus lacking an envelope protein in serum-free medium demonstrated that 1% transduction by Mo-MuLV/Env$^-$ could be demonstrated at a concentration of 0.1 mM CPZ. At 0.2 mM CPZ nearly 100% cell death was observed. The mechanism of protection by serum of the cells against the toxic effects of CPZ is uncertain, although it is possible that the effective CPZ concentration is reduced through its binding to serum proteins.

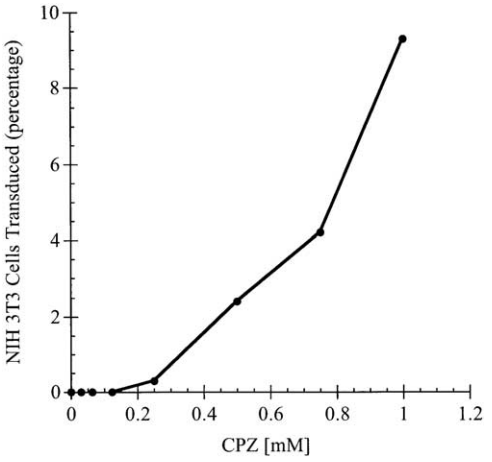

Fig. 3. CPZ concentration dependence of transduction by Mo-MuLV lacking an Env. NIH 3T3 cells were incubated with Mo-MuLV lacking an Env for 4 h prior to treatment with various concentrations of CPZ for 1 min. The level of transduction was determined 32 h after treatment. The data are representative of three independent experiments.

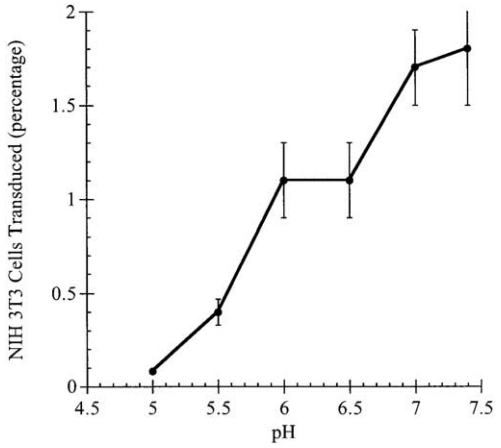

Fig. 4. Dependence of CPZ-induced transduction by Mo-MuLV lacking an envelope protein upon pH. Mo-MuLV lacking an envelope protein was incubated with NIH 3T3 cells for 4 h. The cells were then incubated with 0.5 mM CPZ in medium at the indicated pH for 1 min. The level of transduction was determined 32 h after treatment.

Polybrene does not facilitate CPZ-promoted transduction

Polybrene (hexadimethrine bromide) is a polycationic reagent that facilitates viral infection in vitro. It was of interest to determine whether polybrene affected CPZ-promoted transduction by recombinant Mo-MuLV lacking a viral envelope protein. Virus from gpnlslacZ cells expressing either the wild-type envelope protein or no envelope protein was incubated with NIH 3T3 cells in the presence of various concentrations of polybrene. There was a clear effect of polybrene concentration on transduction by virus bearing the Mo-MuLV Env protein (Fig. 5A). However, the level of transduction by Mo-MuLV/Env$^-$ promoted by 0.5 mM CPZ was independent of polybrene concentration (Fig. 5B), indicating that the effect of polybrene requires the viral glycoprotein and that the mechanisms of membrane fusion promotion by viral Env protein and CPZ are distinct.

Discussion

We demonstrate here that chlorpromazine can promote the transduction of cells by recombinant Mo-MuLV virus particles that do not bear a viral envelope protein. The effect of chlorpromazine concentration and pH upon transduction efficiencies is consistent with a role for CPZ micelle formation in the process (Wajnberg et al., 1988), although there is a distinct possibility that the effective CPZ concentration is reduced by the presence of serum. The concentration dependence of the effects we observed is similar to that seen for the induction of the transition from hemifusion to complete fusion seen in earlier studies (Melikyan et al., 1997). It has previously been demonstrated that the transfection reagent Lipofectin can have a similar capacity to promote transduction by Mo-MuLV particles that lack an

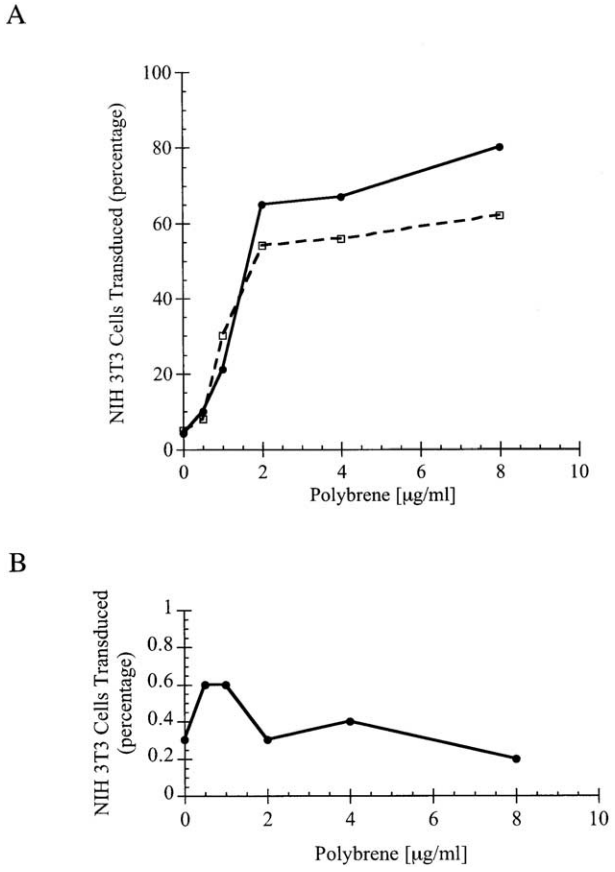

Fig. 5. Polybrene dependence of transduction by virus bearing the Mo-MuLV Env or lacking an Env. NIH 3T3 cells were incubated with virus bearing the Mo-MuLV Env (A) or lacking an Env (B) in the presence of various concentrations of polybrene for 4 h. The NIH 3T3 cells that were incubated with virus lacking an Env were then treated with 0.5 mM CPZ for 1 min. The NIH 3T3 cells that were incubated with recombinant Mo-MuLV bearing Env were treated with 0.5 mM CPZ (dashed line) or untreated (solid line). Thirty-two hours postinfection, the level of transduction was determined. The data are representative of three independent experiments.

envelope protein (Sharma et al., 1997). It is noteworthy that, as we found with CPZ-promoted transduction, Lipofectin-promoted transduction is not enhanced by the presence of polybrene (Sharma et al., 1997). This contrasts with the finding that the promotion of transduction by recombinant retrovirus lacking a viral envelope protein by incubation with vesicles bearing the vesicular stomatitis virus G protein does require polybrene (Sharma et al., 2000). Our data provide further support for the idea that polybrene promotes specifically entry mediated by viral fusion proteins.

The entry of an enveloped virus into a cell can be conceptually divided into a number of steps. There is the primary binding of a virus to a cell surface. This is normally considered the consequence of the binding of a viral glycoprotein to a receptor on a cell surface. However, the process is known to be more complex in a number of viral systems. There may be an interaction between the virus and an attachment factor [CD4 for the human immunodeficiency virus (HIV)] that leads to the association with the authentic receptor (in the case of HIV, a chemokine receptor). The role of the attachment factor may include the reduction of the three-dimensional search for the receptor that actually leads to viral entry to a two-dimensional problem. It has been suggested that the initial association of Mo-MuLV with a cell surface is independent of the Env protein (Pizzato et al., 1999). It may be that such binding is responsible for the interaction that leads to CPZ-promoted transduction.

After binding the conformational changes that lead to membrane fusion must take place. These may transpire after endocytosis of the virus particle and exposure of the viral glycoprotein to the low endocytic pH or they may result directly from receptor-induced conformational changes that occur at the cell surface, as suggested for Mo-MuLV and retroviruses with homologous envelope proteins (Sanders, 2000). It has been demonstrated that CPZ can induce the uptake of a soluble dye into large unilamellar vesicles, perhaps by inducing endovesiculation, although the promotion of fusion between a CPZ micelle and the large unilamellar vesicle can not be excluded (Tedesco and Matile, 1999). It is possible that in our experiments CPZ is inducing endocytosis of the Mo-MuLV particles and that membrane fusion ensues. Alternatively CPZ may promote membrane fusion directly between the virus particle and the cell membrane.

It is known that recombinant retroviruses can incorporate the glycoproteins of other viruses and utilize them for entrance into cells (Jeffers et al., 2002; Sanders, 2002; Sharkey et al., 2001; Yee et al., 1994). It cannot currently be ruled out that a cellular protein that is capable of promoting hemifusion between the viral envelope and the cell membrane is incorporated into the recombinant Mo-MuLV particle and that CPZ merely promotes the transition from hemifusion to full fusion as it is proposed to do in the case of mutants of the influenza virus hemagglutinin (Chernomordik et al., 1998; Markosyan et al., 2000; Melikyan et al., 1997, 1999). We do not have any evidence to support this model and do not favor it.

It should be kept in mind that most of the previous experiments with CPZ concern cell–cell fusion mediated by viral envelope glycoproteins, whereas our studies involved viral–cell fusion. There are potential differences in the processes of cell–cell and virus–cell fusion. Recently, however, it has been argued that the promotion of infection by incubation of a recombinant vesicular stomatitis virus bearing only the ectodomain membrane-proximal, transmembrane, and cytoplasmic domains of the vesicular stomatitis virus G (VSV-G) protein with chlorpromazine provides evidence that the truncated VSV-G protein was able to induce hemifusion in the absence of chlorpromazine (Jeetendra et al., 2002). Our data indicate that this conclusion might not be warranted. Overall, in the interpretation of experiments involving chlorpromazine, it seems prudent to regard it for now as

an agent that can directly promote viral–cell membrane fusion and virus entry.

Materials and methods

Cell lines and cell culture

Mouse NIH 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium (D-MEM; Sigma) with 10% calf serum (CS; Gibco-BRL), streptomycin (0.1 mg/ml), and penicillin (10 U/ml) (PS; Sigma) (D-MEM CS/PS). The gpnlslacZ cells (which produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S-nlsLacZ, a retroviral vector that encodes a nuclear-localized β-galactosidase (Ory et al., 1996; Sharkey et al., 2001)) were grown in D-MEM with 10% fetal bovine serum (FBS; Sigma) and PS (D-MEM FBS/PS).

Viral transduction assays

The penv1min plasmid encoding for the Mo-MuLV envelope protein was transiently transfected into gpnlslacZ (1×10^6) cells following the protocol described previously (Taylor and Sanders, 1999). Medium from the transiently transfected gpnlslacZ cells was removed 32 h posttransfection and filtered through a 0.45-μm filter. It was then incubated with 1×10^6 NIH 3T3 cells in the presence or the absence of 8 μg/ml of hexadimethrine bromide (polybrene) for 4 h. The recombinant virus-containing medium was removed and the NIH 3T3 cells were treated with or without 0.5 mM CPZ (Sigma) in DMEM-CS/PS for 1 min. The cells were then washed with D-MEM CS/PS media and subsequently incubated with fresh D-MEM CS/PS media at 37°C with 5% CO_2 for 32 h before staining with X-gal as described previously (Taylor and Sanders, 1999). The percentage of transduction was determined by counting the number of blue cells with respect to the total number of cells. Each experiment was performed at least twice. The data presented are normally from a single experiment but are representative.

Chlorpromazine studies

Virus from gpnlslacZ cells (1×10^6) that were transiently transfected with penv1min, the plasmid encoding the wild-type Mo-MuLV envelope protein, or that were mock transfected was collected and used to infect NIH 3T3 cells in the presence or the absence of various concentrations of polybrene for 4 h at 37°C with 5% CO_2. Four hours postinfection the cells were treated with various concentrations of chlorpromazine (CPZ, Sigma) for 1 min in D-MEM CS/PS media at the appropriate pH. The cells were washed in fresh D-MEM CS/PS media and subsequently incubated at 37°C with 5% CO_2 for 32 h before staining with X-gal. Each experiment was performed at least twice. The data presented are normally from a single experiment but are representative. Incubation of the cells with virus in serum-free medium (D-MEM) was conducted similarly, but toxicity was observed when CPZ concentrations higher than 0.1 mM were present.

Fluorescence-activated cell sorting of transduced cells

NIH 3T3 cells transduced with MFG.S-nlsLacZ were passaged for 2 weeks. The cells were then stained with the fluorogenic β-galactosidase substrate FDG. The cells were suspended at 10^7 cells/ml in staining media (PBS, 4% FBS, 10 mM HEPES, pH 7.4, 300 μM chloroquine). After warming of the suspended cells at 37°C for 20 min, the cells were incubated with 2 mM FDG at 37°C for 1 min. The reaction was stopped by the addition of staining medium containing 1.5 μM propidium iodine and 300 μM chloroquine at 4°C. The stained cells were kept on ice during sorting. Transduced cells were sorted by fluorescence-activated cell sorting with a Coulter Epics Elite cell sorter using a 630-nm band pass filter and a 488-nm air-cooled argon laser. The cells that were positive for fluorescence were grown for an additional 4 weeks before detection of retroviral DNA was attempted.

Detection of retroviral DNA in stable transduced cells

DNA from nontransduced and sorted transduced NIH 3T3 cells was prepared by phenol:chloroform extraction. A 1.9-kb C terminal portion of the *nlslacZ* gene was detected by two rounds of amplification by the polymerase chain reaction (PCR) utilizing the Taq DNA Core Polymerase System (Qiagen) with the following primers: forward, 5′ GATTGAAGCAGAAGCCTGCG 3′; reverse, 5′ GTTTCCATCAGTTGCTGTTG 3′. Amplification of the Rapsn acetylcholine receptor-associated protein (43K) gene was used as a control (Williams et al., 2003). PCR products were resolved by agarose gel electrophoresis.

Acknowledgments

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