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α-Complementation assay for HIV envelope glycoprotein-mediated fusion

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Abstract

The fusion reaction mediated by viral envelope glycoproteins proceeds through an ordered series of conformational changes in the envelope glycoprotein. Fusion inhibitors have been developed that target glycoprotein subunits, arresting the reaction at different points in the process. We report the development of a novel method for detecting viral glycoprotein-mediated fusion that is based on the principle of α-complementation of β-galactosidase. The method is simple, accurate, has a high signal-to-noise ratio, is suited for high-throughput screening, and does not require new transcription or protein synthesis. Cells expressing a viral envelope glycoprotein and the N-terminal α fragment of β-galactosidase were mixed with cells expressing the C-terminal β-galactosidase fragment, CD4, CCR5, or CXCR4. Fusion was detected after 30 min and continued to increase to very high levels for more than 5 h. The assay was used to examine the temperature dependence of fusion and the effect of coreceptor and glycoprotein density on inhibitor activity.

Keywords: Membrane fusion; Fusion assay; Envelope; Entry; HIV; β-galactosidase

Introduction

Enveloped viruses enter target cells via a fusion reaction mediated by the viral envelope glycoprotein (reviewed in Chan and Kim, 1998; Eckert and Kim, 2001). Entry is initiated when the surface component of the envelope glycoprotein (SU) attaches to its receptor on the target cell surface. In many enveloped viruses, attachment induces an ordered series of conformational rearrangements in SU and the transmembrane component (TM) that finally result in the fusion of the lipid bilayer of the virus with the target cell, either at the plasma membrane or in a low pH endosome.

HIV-1 attachment is mediated by the interaction of the viral gp120 SU and its receptor on the target cell, CD4. This binding induces a conformational change in gp120 that exposes a binding site for a coreceptor, usually either of the chemokine receptors CCR5 or CXCR4 (Berger et al., 1999; Weiss, 2002). Coreceptor binding triggers rearrangement of the gp41 TM from its compact native conformation into an extended pre-hairpin intermediate, in which the hydrophobic amino-terminal peptide of gp41 inserts into the target cell plasma membrane (Doms and Moore, 2000). The gp41 sequence contains two heptads repeats (HR1 and HR2) that exist as separate triple helical coiled coils. After attachment, the two domains are brought into proximity with one another to form a single six-helix bundle in which the C-terminal helices of HR2 fit into grooves on the outside of the N-terminal coiled coil of HR1 (Chan et al., 1997; Weissenhorn et al., 1996). Accumulation of these six helical bundles allows the formation of a fusion pore that ultimately results in fusion of the viral and cellular membranes (Melikyan et al., 2000).

Virus entry is a promising target for the development of novel therapeutics (reviewed in Eckert and Kim, 2001; Moore and Stevenson, 2000). The glycoprotein and the receptor proteins are exposed at the viral and cell surfaces, and thus compounds that inhibit viral entry need not be membrane soluble. In addition, the coreceptors are encoded by cellular genes and so are not susceptible to mutations that

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would cause resistance to antiviral drugs. Several classes of entry inhibitors have been described and are in various stages of development (reviewed in Blair et al., 2000; D'Souza et al., 2000). These include (i) small molecule coreceptor antagonists of CCR5 (Tak779 (Baba et al., 1999), AD101 (Trkola et al., 2002), and SCH-C (Strizki et al., 2001)) or CXCR4 (AMD3100 (Schols et al., 1997)); (ii) chemokine analogues with modified amino-termini (reviewed in Blanpain et al., 2002); (iii) the receptor mimic, soluble CD4 (Allaway et al., 1995); (iv) peptide fusion inhibitors T21 (Wild et al., 1992), T20 (Kilby et al., 1998), and C34 (Chan et al., 1997; Jiang et al., 1993); and (v) neutralizing antibodies that bind gp120 or gp41 (reviewed in Burton, 2002).

The peptide fusion inhibitors are derived from gp41 HR1 (T21) or HR2 (T20 and C34). These peptides are active at nanomolar concentrations and are thought to block entry of R5 and X4 isolates by targeting a transient intermediate conformation of gp41 that forms upon CD4/coreceptor contact (Chan et al., 1997; Jiang et al., 1993). They are believed to bind HR1 or HR2 in the pre-hairpin intermediate, where they interfere with conversion to the hairpin conformation and thus block the formation of the six helical bundles required to form the fusion pore (Chan and Kim, 1998, and references therein).

HIV-1 envelope glycoprotein-mediated entry and fusion have been measured by several methods. These include single replication cycle viruses that contain a reporter gene (Connor et al., 1995), virions loaded with β-lactamase (Cavrois et al., 2002), and cell–cell fusion assays (Lineberger et al., 2002; Nussbaum et al., 1994). In cell fusion assays, cells expressing the glycoprotein are mixed with cells that express CD4 and CCR5 or CXCR4. Interaction of the glycoprotein with the receptor and coreceptor on target cells triggers fusion of the cells, which is quantitated by microscopic counting of syncytia or quantitation of a reporter gene transactivation.

Here we report the development of a novel cell–cell fusion assay to measure viral glycoprotein-mediated fusion that represents a significant advance over current methods. The assay is based on the unique property of β-galactosidase fragments to complement in trans, a phenomenon that has been termed α-complementation (Ullmann et al., 1967, and references therein). β-galactosidase is enzymatically active as a tetramer and has the unique property that it can be divided into two fragments, an N-terminal α peptide (amino acids 1–80) and a C-terminal ω fragment (amino acids 80–1023) that complement in trans (Moosmann and Rusconi, 1996). The fragments are enzymatically inactive separately, but when expressed together in cells, enzymatic activity is reconstituted. α-Complementation of β-galactosidase was initially found in *Escherichia coli* (Blakely et al., 2000; Rossi et al., 1997) but can also occur in mammalian cells (Moosmann and Rusconi, 1996), and has been used to study protein–protein interactions (Rossi et al., 2000).

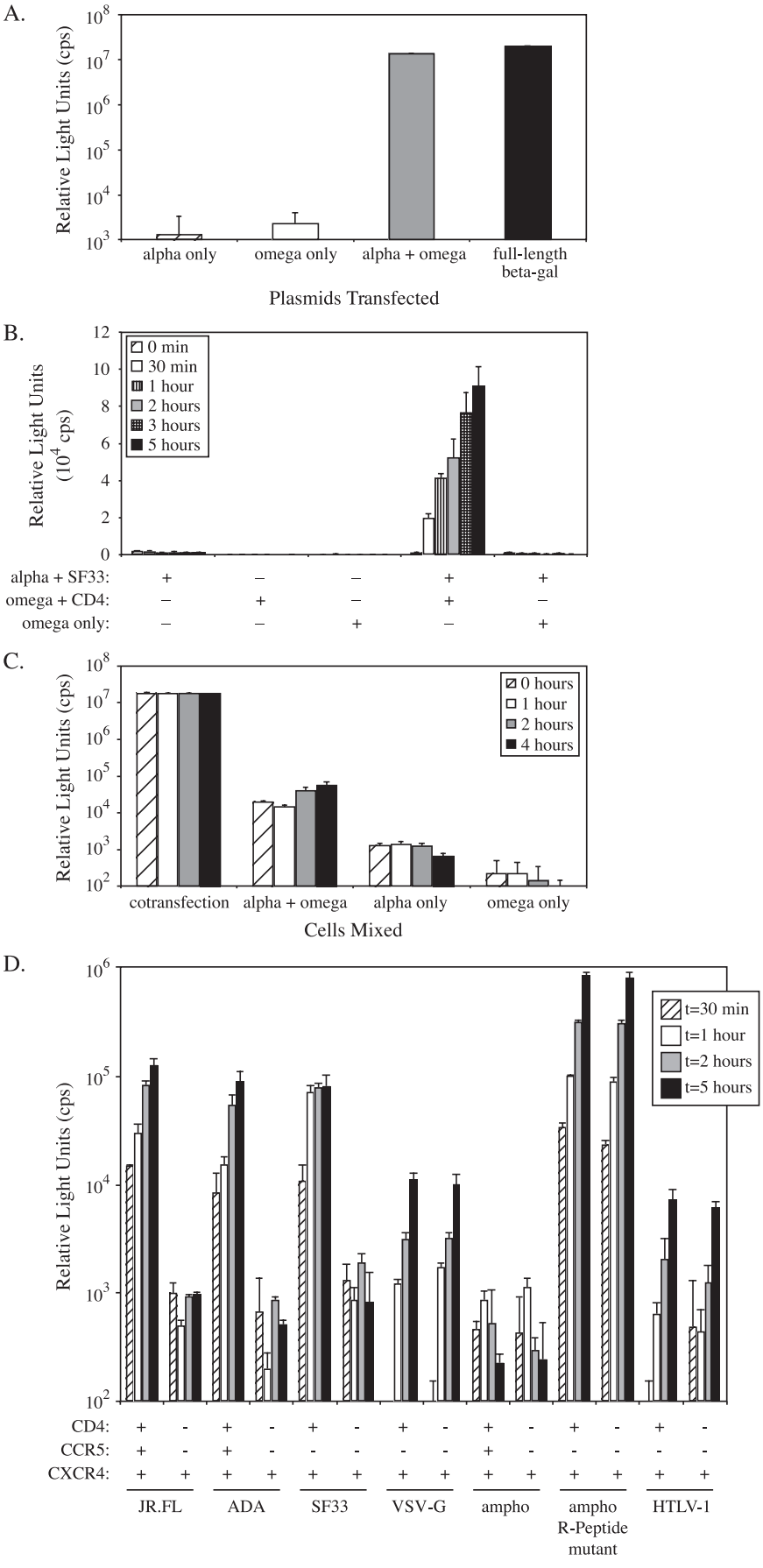
We applied the α-complementation principle to detect viral glycoprotein-mediated fusion. This allowed measurement of fusion without the need for preloading the target cell with an enzymatic substrate or relying on downstream events such as syncytia formation or reporter gene activity. In addition, because the assay does not involve microscopic enumeration of fusion events, it is objective and rapid. It offers increased resolution of the timing of early fusion events in comparison to many other common fusion assays, with the exception of the β-lactamase-based assay. The method can be generalized to other viral fusion systems or other biological phenomena that involve cell–cell fusion and is particularly well suited to high-throughput screening for small molecule entry inhibitors of HIV or other enveloped viruses. We have used the assay to understand parameters of HIV fusion, temperature optima, and activities of fusion inhibitors.

Results

To demonstrate that α-complementation was active in cells that support virus-mediated fusion, 293T cells were transfected with expression vectors that expressed β-galactosidase α (pCMVα) or ω (pCMVω) (Moosmann and Rusconi, 1996). For comparison, 293T cells were also transfected with pCMV-β-gal, a vector expressing the full-length enzyme. Cells transfected with α and ω expression vectors separately contained very low β-galactosidase activity. In contrast, when the two vectors were cotransfected, they complemented to yield highly active enzyme (Fig. 1A). The α-complemented enzyme was nearly as active as the native enzyme, resulting in β-galactosidase activity more than 1000-fold above background levels.

To apply this phenomenon to the detection of viral envelope glycoprotein-mediated fusion, 293T cells were transiently cotransfected with vectors that expressed the CXCR4-specific HIV-1 SF33 envelope glycoprotein, HIV-1_{BRU} Rev and β-galactosidase α. Two days posttransfection, the cells were mixed with an equal number of 293T cells transfected to express CD4, and β-galactosidase ω. β-galactosidase activity was then measured over the next 5 h (Fig.

Fig. 1. Demonstration of α-complementation. (A) 293T cells were transfected with pCMV-α, pCMV-ω, or pCMV-β-gal. β-galactosidase activity was measured in triplicate samples 48 h posttransfection. (B) 293T cells transfected with pCMV-α, Rev expression plasmid, and HIV-1_{SF33} envelope glycoprotein expression plasmid were mixed in triplicate with 293T cells stably expressing ω alone, or ω and CD4. Each cell type alone, as well as the two cell mixtures, was incubated at 37°C for the indicated times after which β-galactosidase activity was measured. (C) 293T cells transfected as in A were mixed in 1:1 ratios 2 days posttransfection. The cell mixtures were pelleted by low speed centrifugation and fused with 50% (w/v) polyethylene glycol for 5 min. The cells were then washed and incubated at 37°C for the indicated times, after which β-galactosidase activity was measured. (D) Glycoprotein and α-expressing cells were prepared as in B. The target cells stably expressed ω and CXCR4. Where indicated, CD4 was stably expressed and CCR5 was transiently expressed. Cells were mixed, incubated, lysed, and β-galactosidase activity measured as in B.


A.
Relative Light Units (cps)
alpha only
omega only
alpha + omega
full-length beta-gal
Plasmids Transfected
B.
Relative Light Units (10^4 cps)
0 min
30 min
1 hour
2 hours
3 hours
5 hours
alpha + SF33:
omega + CD4:
omega only:
C.
Relative Light Units (cps)
0 hours
1 hour
2 hours
4 hours
cotransfection
alpha + omega
alpha only
omega only
Cells Mixed
D.
Relative Light Units (cps)
t=30 min
t=1 hour
t=2 hours
t=5 hours
CD4:
CCR5:
CXCR4:
JR.FL
ADA
SF33
VSV-G
ampho
ampho R-Peptide mutant
HTLV-1

1B). β-galactosidase activity was first detected 30 min after mixing and continued to develop over time to a level 100-fold higher than control cultures that lacked one of the components. Artifactual explanations for the β-galactosidase activity were ruled out by additional controls. For example, polyethylene glycol (PEG)-mediated fusion of 293T cells transiently expressing β-galactosidase α with 293T cells expressing β-galactosidase ω resulted in a signal that appeared immediately after the PEG was removed, and increased less than 3-fold over the next 4 h (Fig. 1C), an increase that was probably due to new protein synthesis rather than additional cell fusion. Thus, the α and ω fragments assemble very rapidly, and the increase in β-galactosidase signal during the viral glycoprotein-mediated fusion time course (Fig. 1B) is due to increased membrane fusion over time. This accumulation of β-galactosidase activity was CD4-dependent. When CD4 was not expressed on target cells, β-galactosidase activity was close to background levels. This finding demonstrated that there was no significant amount of α-complementation that occurred due to α and ω proteins that had leaked from cells, and that there was no significant amount of spontaneous cell–cell fusion. In addition, the signal was not due to artifactual α-complementation that occurred upon lysis of cultures in the assay buffer. Although free α and ω are released upon lysis of cells, the lysis buffer appeared to prevent postlysis association of the two fragments, as well as postlysis dissociation of preformed active enzyme complexes. Incubation of the cells before mixing with the protein synthesis inhibitor cycloheximide did not prevent α-complementation (data not shown). This result demonstrated that complementing proteins exist in the cells before fusion.

In principle, this method could be used to detect fusion mediated by other viruses that contain fusogenic envelope glycoproteins. To extend the findings to other viruses, we tested glycoproteins from the R5 HIV-1 isolates ADA and JR.FL, as well as glycoproteins from three other viruses, vesicular stomatitis virus (VSV), human T cell leukemia virus (HTLV), and amphotropic murine leukemia virus (A-MuLV). The fusogenicity of the A-MuLV glycoprotein is regulated by the length of its cytoplasmic tail (Rein et al., 1994), so both the full-length glycoprotein and a cytoplasmic tail deletion mutant were tested. 293T cells were cotransfected with expression vectors for each glycoprotein, HIV-1 Rev, and the α fragment. The transfected cells were mixed with target cells that stably expressed ω, CD4, and CXCR4, and transiently expressed CCR5 where indicated. The JR.FL, ADA, and SF33 glycoproteins were all CD4-dependent and required expression of the appropriate coreceptor (Fig. 1D). The other three envelopes were all CD4-independent, as expected. Full-length A-MuLV glycoprotein did not give a signal but the cytoplasmic tail truncation was highly active (about 10-fold more than JR.FL or ADA), demonstrating the tight regulation of fusogenicity by the cytoplasmic tail. VSV-G and HTLV-1 envelopes were also active in the assay, although they only reached 10% of the activity of R5 HIV-1. Despite the fact that VSV-G entry occurs in low pH endosomes, acidification of the medium did not increase the signal in this assay (data not shown).

Earlier reports (Golding et al., 2002; Melikyan et al., 2000) have described pronounced temperature optima for HIV-1 glycoprotein-mediated fusion. In these studies, fusion was found to occur at 37°C, but not at lower temperatures such as 22 or 31.5°C. At these reduced temperatures, Env is thought to bind to CD4 and coreceptor and proceed to the pre-hairpin conformation, where it becomes trapped. These studies were done by manually counting the number of syncytia formed over time at various temperatures. We were interested to reexamine the temperature dependence of fusion using the α-complementation assay to directly detect the reaction. Cells expressing X4 SF33 or R5 ADA glycoproteins were tested in the fusion assay over a range of temperatures for 4 h (Fig. 2A). Fusion occurred efficiently from 23 to 40°C and was optimal at 30°C. This temperature optimum was not due to differing efficiencies of assembly of α and ω over the temperature range because PEG-mediated fusion over the same range of temperatures failed to demonstrate significant temperature dependence (data not shown). The activities of these two envelope glycoproteins were also tested at several time points ranging from 1 to 4 h (Fig. 2B). Reactions performed on ice or at 20°C gave undetectable signals over the time span of the experiment, whereas reactions performed at both 31.5 and 37°C gave high signals. For both envelope glycoproteins, reactions incubated at room temperature resulted in undetectable levels of fusion until they had been allowed to fuse for 4 h, at which point the signals rose slightly to approximately 8- to 10-fold above background.

The connection between temperature and peptide fusion inhibitor activity was also examined. The fusion assay was performed in parallel at the two temperatures that gave high signals in the previous experiment, both in the absence and presence of a low concentration of the peptide fusion inhibitor T-20. The concentration of T-20 used (6.25 μg/ml) was determined empirically by assaying effectiveness of the inhibitor at 37°C over a wide range of concentrations (data not shown). The concentration selected was the highest concentration at which little to no effect of the compound could be observed at 37°C. This is demonstrated in Fig. 2C, where the effect of T-20 on the reaction at 37°C is minimal and disappears after the reaction has proceeded for 2 h. The effect of the same concentration of T-20 on the reaction performed at 31.5 °C is more dramatic and results in a reduction in the progress of the fusion reaction to about half of the signal generated by the reaction done in the absence of T-20.

Several categories of fusion inhibitors were tested for activity in the α-complementation fusion assay. IgG-CD4 immunoadhesion was tested for inhibitory activity by performing parallel fusion reactions for 4 h at 37°C in the absence and presence of 5-fold dilutions of inhibitors (Fig. 3A). The cells used were 293T cells transfected with the α

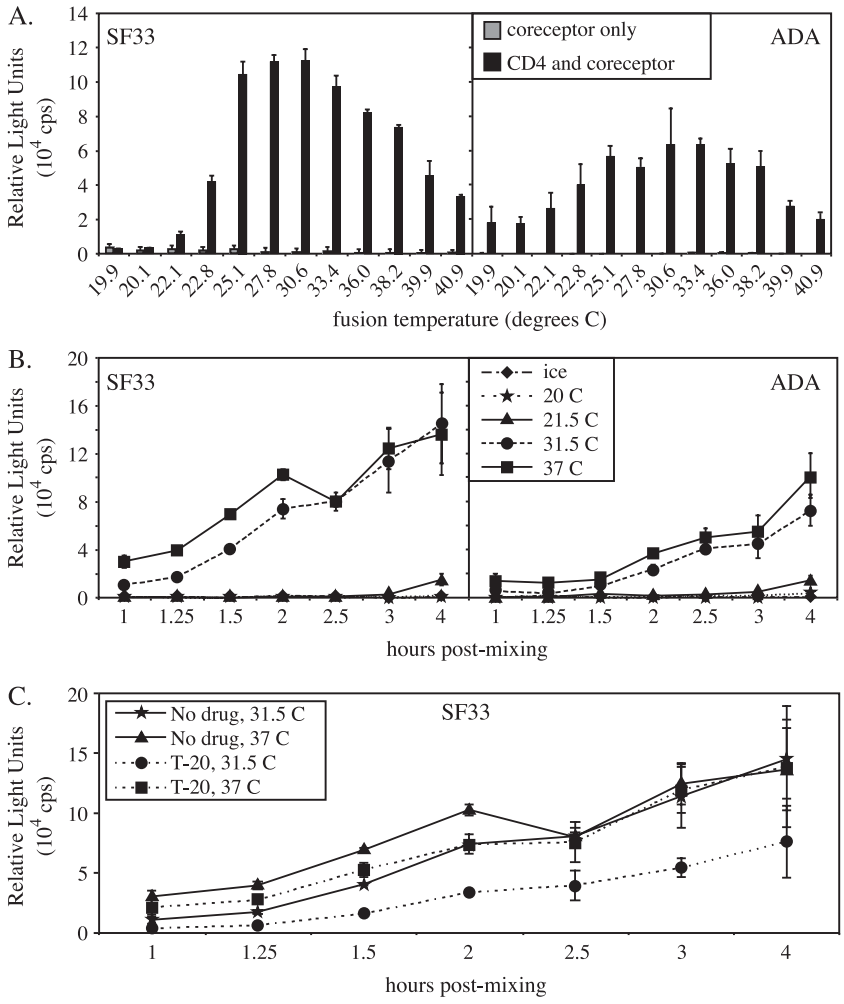


Fig. 2. Temperature effects. (A) 293T cells expressing α, Rev, and SF33 or ADA glycoprotein were mixed with 293T target cells that stably expressed ω, CXCR4, and CD4 where indicated. The target cells mixed with the ADA envelope also transiently expressed CCR5. Cell mixtures were incubated at the indicated range of temperatures and β-galactosidase activity was measured after 4 h. (B) Cells were prepared and mixed as in A and were then incubated at the indicated temperature for the indicated times. CD4 was expressed on all target cells. (C) T-20 (6.25 μg/ml) was added to the envelope-expressing cell before mixing.

fragment, HIV-1 Rev, and one of three HIV envelopes, while target cells were 293T cells stably expressing the ω fragment and CD4 (for the SF33 reactions) and 293T cells stably expressing the ω fragment and CD4 and transiently transfected with low levels of CCR5 (for ADA and JR.FL reactions, 200 ng pcDNA-CCR5 per 10 cm plate). sCD4-IgG was capable of inhibiting both R5- and X4-tropic HIV envelopes, though fairly high concentrations (20–100 μg/ml) were necessary to completely block fusion. Interestingly, low concentrations of CD4-Ig appeared to enhance fusogenicity of the SF33 envelope, while higher concentrations were inhibitory. This effect was likely due to an sCD4-induced conformational change in the envelope glycoprotein that allowed it to more readily proceed to CXCR4 binding.

Four different small-molecule CCR5 inhibitors and one CXCR4 inhibitor were tested for activity in the assay in a similar fashion as for sCD4. The target cell for the CCR5 inhibitors was a HeLa cell line stably expressing the ω fragment, CD4, and low levels of CCR5. Reactions performed with this cell line gave lower signals than reactions performed with cells transiently transfected with CCR5, but the stable cell line gave more consistent data in the assay and was thus used here. The most potent CCR5 inhibitor in the assay was TAK779, followed by the Merck compound, Schering C, and finally the isomer of Schering C. AMD-3100 was an active inhibitor of CXCR4-mediated fusion over a similar range of concentrations. All four inhibitors were active at concentrations extending down into the mid-nanomolar range. Similar titration curves were generated for two neutralizing antibodies against CD4 and CCR5, Leu3A, and 2D7. Both antibodies were able to completely block fusion at concentrations of 2–10 μg/ml.

The titration curves that were generated (Fig. 3A) were used to estimate the IC_{50} of each of the CCR5 inhibitors in the fusion assay. Similar titration curves were generated for each compound using pseudotyped single-cycle luciferase reporter viruses, a well-established assay for Env-mediated

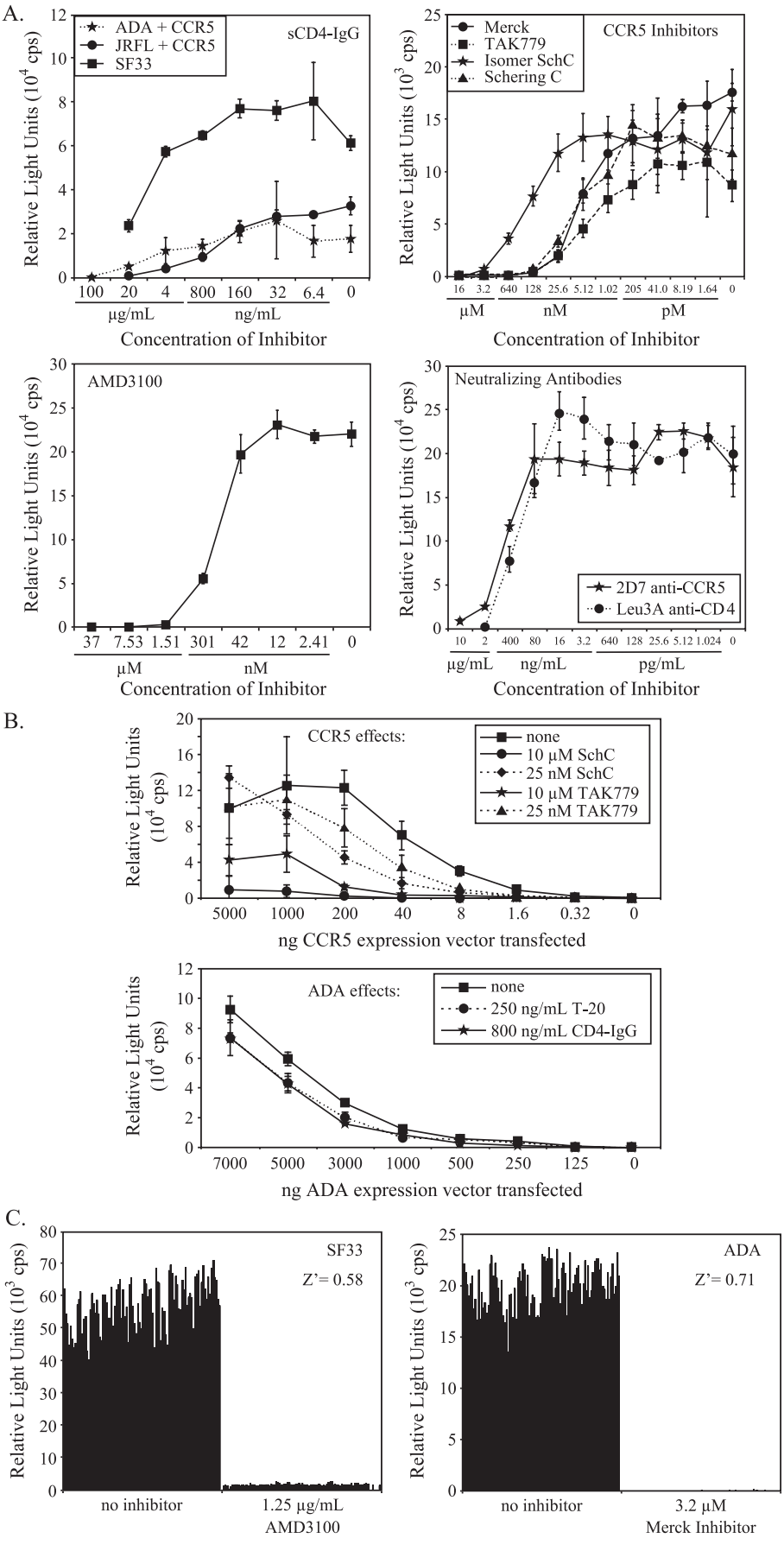

A.
ADA + CCR5
JRFL + CCR5
SF33
sCD4-IgG
Relative Light Units (10^4 cps)
100 20 4 800 160 32 6.4 0
µg/mL ng/mL
Concentration of Inhibitor
Merck
TAK779
Isomer SchC
Schering C
CCR5 Inhibitors
Relative Light Units (10^3 cps)
16 3.2 640 128 25.6 5.12 1.02 205 41.0 8.19 1.64 0
µM nM pM
Concentration of Inhibitor
AMD3100
Relative Light Units (10^4 cps)
37 7.53 1.51 301 42 12 2.41 0
µM nM
Concentration of Inhibitor
Neutralizing Antibodies
2D7 anti-CCR5
Leu3A anti-CD4
Relative Light Units (10^4 cps)
10 2 400 80 16 3.2 640 128 25.6 5.12 1.024 0
µg/mL ng/mL pg/mL
Concentration of Inhibitor
B.
CCR5 effects:
none
10 µM SchC
25 nM SchC
10 µM TAK779
25 nM TAK779
Relative Light Units (10^4 cps)
5000 1000 200 40 8 1.6 0.32 0
ng CCR5 expression vector transfected
ADA effects:
none
250 ng/mL T-20
800 ng/mL CD4-IgG
Relative Light Units (10^4 cps)
7000 5000 3000 1000 500 250 125 0
ng ADA expression vector transfected
C.
SF33
Z'= 0.58
Relative Light Units (10^3 cps)
no inhibitor
1.25 µg/mL AMD3100
ADA
Z'= 0.71
Relative Light Units (10^3 cps)
no inhibitor
3.2 µM Merck Inhibitor

Table 1
Comparison of IC_{50} values derived from the α-complementation assay and single-cycle luciferase virus infection

Inhibitor	Estimated IC_{50}: fusion assay[a] (nM)	Estimated IC_{50}: luciferase virus inhibition[b] (nM)
Merck compound	5	10
TAK779	10	8
Schering C	20	5
Isomer Schering C	100	2

[a] Values for IC_{50} were estimated from the curves shown in Fig. 3A.
[b] Inhibition curves were generated by adding each of the inhibitors to a single-cycle infection with NL4-3 $R^-E^-Luc^+$ virions pseudotyped with the ADA envelope. Values for IC_{50} were estimated as for the α-complementation assay.

virus–cell fusion and entry, and the IC_{50} values were estimated as for the fusion assay and the values compared (Table 1). Only one of the four inhibitors compared in this fashion showed a large difference in IC_{50} as measured by the two different assays, which might reflect differences between membrane fusion and infection.

The effects of envelope expression levels as well as coreceptor expression levels on the efficiency of fusion inhibition were examined (Fig. 3B). In each case, cells were transfected with decreasing amounts of the appropriate expression vector, and multiple fusion assays were performed both in the absence of an inhibitor and in the presence of a high or low concentration of two different inhibitors. In the case of CCR5 expression levels, low concentrations of the inhibitor (that had little effect on fusion when a large amount of CCR5 was transfected) showed an inhibitory effect on fusion when smaller amounts of CCR5 were transfected, most noticeably between 8 and 200 ng. Conversely, varying the envelope expression levels did not result in a similar effect. Concentrations of the inhibitor that inhibited fusion by about 15% when a large amount of Env was expressed were only able to inhibit approximately the same proportion of the signal as envelope expression decreased. FACS analysis of similarly transfected 293T cells showed that transfection of increasing amounts of CCR5 expression plasmid resulted in higher levels of CCR5 expression per cell rather than an overall increase in the proportion of transfected cells (data not shown).

Application of the assay to high-throughput inhibitor screening was investigated using the method of Zhang et al. (1999) to define a factor Z′, which is a measure of the frequency with which false positive hits are detected over a large number of measurements. The fusion reaction was adapted to a microplate format and tested with an X4-tropic glycoprotein in the absence or presence of IC_{95} of the X4 inhibitor AMD3100, and with an R5-tropic glycoprotein in the absence or presence of IC_{95} of Merck's small-molecule R5 inhibitor (Fig. 3C). The mean and standard deviation of the signal under each of the four conditions was calculated and used to determine the Z′ factor for each envelope. The SF33 envelope resulted in a Z′ of 0.58, while the ADA envelope gave a value of 0.71. Each of these values is above 0.5, a figure that is considered sufficient for screening. Further optimization, for example, by the use of automated liquid handling robotics, should further increase this value.

Discussion

We report here on the development of a novel assay based on the principle of α-complementation of β-galactosidase for the detection of viral envelope glycoprotein-mediated fusion. The method has advantages over current methods with respect to simplicity, accuracy, rapidity, and objectivity. It allows examination of early events that occur during fusion because it neither requires completion of any post-entry steps in the viral life cycle, nor does it depend on de novo protein synthesis of reporter genes. We found that HIV-mediated fusion was optimal at reduced temperatures and that inhibitor activity was dependent on coreceptor expression level, consistent with the findings of Reeves et al. (2002).

The peptide inhibitors are an important class of fusion inhibitors. These molecules act not on the native protein, but on conformational intermediates formed upon CD4–coreceptor binding (Chan et al., 1997; Jiang et al., 1993). Low temperature increases their activity presumably by stabilizing envelope glycoprotein conformational intermediates, consistent with the earlier findings of Golding et al. (2002). Because α-complementation was robust at the suboptimal temperatures that expose these intermediates, screening at reduced temperature could favor the identification of small molecules that mimic the peptide inhibitors. We did not find that fusion was trapped at 31.5 °C. In our assay, with the particular envelope glycoproteins used, we rather found that fusion was optimal at reduced temperatures. Blocking of fusion required temperatures close to 22 °C.

The inhibitors tested were generally of similar potency in the fusion assay as compared to reporter virus infection assays. This suggested that cell–cell fusion accurately models the fusion between virus and cell. Thus, the α-complementation assay is suitable for the identification of compounds that target the envelope glycoprotein or the

Fig. 3. Activity of entry inhibitors. (A) Fusion inhibitors were serially diluted in DMSO (CCR5 inhibitors) or PBS (sCD4, AMD3100, and antibodies). Two microliters of each inhibitor were added to the envelope-expressing cell (sCD4) or the CD4–coreceptor-expressing cell (CCR5 inhibitors, AMD3100, and antibodies) before cell mixing. All samples were incubated at 37 °C for 4 h. (B) Serial dilutions of pcDNA-CCR5 or pSV-ADA were transfected into 293T cells and tested in the fusion assay both in the absence and presence of an inhibitor as indicated. (C) 96 parallel fusion reactions were performed in the absence and presence of IC_{95} concentrations of entry inhibitors, and the resulting signal and background averages and standard deviations were used to quantify the effectiveness of the assay for high-throughput screening.

HIV-1 receptor and coreceptor (CD4 and CCR5 or CXCR4). Entry inhibitors that were active in the assay included CCR5 and CXCR4 antagonists, the peptide inhibitor T-20, and antibodies against the receptor and coreceptor. Screening with the assay could detect compounds that target any of the three surface proteins. The target of active compounds can then be distinguished in a secondary screen. Compounds can be initially classified by whether they act on the envelope-expressing cell or the CD4–coreceptor-expressing cell.

The simplicity and accuracy of the α-complementation method are well suited to high-throughput screening for fusion and entry inhibitors. Because fusion is detected without relying on activation of a reporter gene and because the complementing proteins preexist in the cells, compounds that have global nonspecific effects on transcription, translation, or protein trafficking are not expected to confound the analysis. This method can be applied to studies on other viral glycoproteins in addition to HIV-1. Glycoproteins from amphotropic MLV, VSV, and HTLV-1 were active in the assay. In addition, the assay may be applicable for detecting fusion in nonviral systems, such as the SNRPs that control trafficking of intracellular vesicles in cells (Skehel and Wiley, 1998).

Materials and methods

Expression vectors and cell lines

Vectors for expression of the α fragment (pCMV-α, formerly pSCTZ-α-N85), the omega fragment (pCMV-ω, formerly pSCTZ-omega), and full-length β-gal (pCMV-β-gal, formerly pSCTZ) (Moosmann and Rusconi, 1996), the HIV-1_{BRU} expression vector, pRSV-Rev (Hope et al., 1990), and envelope expression vectors (pSV-ADA and pSV-JRFL (Deng et al., 1996), pCAGGS-SF33 (York-Higgins et al., 1990), pCMV-G (Yee et al., 1994), pMLVampho (Deng et al., 1996), pRR186 (Rein et al., 1994), and HTLV-1 env (Landau et al., 1991)) were previously described. 293T and HeLa cells that stably expressed omega were generated by cotransfection with pCMV-ω and pSV-hygro. Individual cell clones were expanded and tested by transfection with pCMV-α or by testing for activity in the α-complementation assay. ω-Expressing cell clones were transduced to express CD4 (Maddon et al., 1985) and CCR5 using pBABE retroviral vectors (Morgenstern and Land, 1990; Onishi et al., 1996). Transient expression of CD4 was achieved by transfection of pcDNA-CD4 (Lee et al., 1999), and different levels of CCR5 were achieved by transfection of a 5-fold serial dilution of pcDNA-CCR5 (Deng et al., 1996).

Measurement of α-complementation in human cell lines

Cells were cultured in DMEM/10% fetal bovine serum/10 mM HEPES supplemented with penicillin and streptomycin. Puromycin (1 μg/ml) was included for passage of retroviral vector-transduced cell lines. 293T cells (2.5×10^6) were seeded in 10-cm dishes and transfected with 10 μg pCMV-α, 10 μg pCMV-ω, or 10 μg pCMV-β-gal (Moosmann and Rusconi, 1996) using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were removed from plates with trypsin–EDTA (Gibco) and dispensed in triplicate into thin-walled PCR strip-tubes (Phenix Research Products) at 5×10^5 cells/tube. The cells were lysed in 100 μl lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100) and 10 μl was used for luminescent measurement of β-galactosidase activity using GalactoStar reagents (Applied Biosystems) and a TopCount microplate luminometer.

For polyethylene glycol-mediated fusion, the cells were transfected with α and ω expression vectors. Two days later, the cells were mixed at a 1:1 ratio (5×10^5 total cells/tube) and pelleted by low speed centrifugation. The cells were fused by replacing the medium with 100 μl of 50% (w/v) polyethylene glycol (Sigma, average molecular weight: 3350) prewarmed to 37°C. After 5 min at room temperature, the PEG was removed by washing twice with 4°C medium and replaced with fresh medium. Following 37°C incubation for the indicated times, β-galactosidase activity was measured as above.

Measurement of envelope glycoprotein-mediated fusion

293T cells (2.5×10^6) were transfected by lipofection with pCMV-α, pRSV-Rev, and envelope expression vector (7 μg each). Target 293T or HeLa cells either stably expressed the ω fragment and the desired receptors, or were lipofected with pCMV-ω, pcDNA-CD4, and pcDNA-CCR5. Two days posttransfection, cells were removed from culture dishes with PBS/5 mM EDTA and adjusted to 5×10^6 cells/ml in medium. Cells expressing the envelope glycoprotein (50 μl) were mixed with an equal volume of target cells in thin-walled PCR strip-tubes, lightly pelleted by a 10-s centrifugation, and incubated in a water bath or a thermal cycler (GeneAmp 9700, Applied Biosystems, or Mastercycler Gradient, Eppendorf). For experiments in which antibodies or inhibitors were included, these were added to the appropriate target cell type before the cells were mixed. After incubation for the indicated times, β-galactosidase activities were measured as described above.

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