Quantification of virus-envelope-mediated cell fusion using a tetracycline transcriptional transactivator: fusion does not correlate with syncytium formation

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Abstract

Cell fusion occurs in many cellular processes and viral infections. We developed a new, quantitative cell fusion assay based on the tetracycline-controlled transactivator (tTA)-induced expression of a luciferase reporter gene. The assay is objective, sensitive, linear over 2–3 orders of magnitude, amenable to microtiter-plate format, and generalizable to study fusion mediated by a variety of genes. Applied to HIV and MLV, cell fusion paralleled virus entry in terms of co-receptor requirements, need for post-translational processing of envelope, and complementation of SU mutations by soluble receptor-binding domain. However, biochemically measured fusion did not correlate with syncytia detected by standard light microscopy. When the assay indicated cell fusion occurred but overt syncytia were not observed, confocal microscopy using fluorescent protein markers showed that fusion was limited mainly to pairs of cells. Such nonprogressive cell fusion suggests that post-translational processing of envelope may be altered in heterokaryons co-expressing envelope and receptor.

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Introduction

Cell fusion is important in many developmental processes such as formation of placenta, muscle, and bone (Blobel et al., 1992; Mi et al., 2000; Vignery, 2000; Yagami-Hiromasa et al., 1995), in inflammatory conditions (Anderson, 2000), and in viral infections. In most of these cases, the fusing cells form multinucleate giant cells, i.e., syncytia, easily detectable by light microscopy. However, cell fusion can be subtler, involving only two cells, yet still have enormous consequences, as in fertilization or formation of somatic cells hybrids. Cell fusion is important in research and bioengineering applications such as somatic cell genetics and monoclonal antibody production, and recently has been found to be responsible for some cases of stem cell transdifferentiation (Terada et al., 2002; Ying et al., 2002).

Cell fusion mediated by virus envelope genes provides a model to study the process by which enveloped viruses enter cells. Virus-induced cell fusion may also be responsible for some forms of virus cytopathicity in vivo. Virus-induced syncytium formation has been used to quantify viral infection (Rowe et al., 1970), although the ability to form syncytia is often restricted to particular cell types or virus strains. Little is known about why some cells readily form syncytia while others do not, although they may be equally susceptible to virus infection, or why syncytium formation can be associated with point mutation in the receptor binding domain of viral envelope genes (Chung et al., 1999; Watkins et al., 1997), or shortening the cytoplasmic tail of viral envelope TM genes (Rein et al., 1994; Spies and Compans, 1994; Zingler and Littman, 1993). As a step toward understanding the relationship between virus-mediated cell fusion and syncytium formation, we developed a...
biochemical assay for virus-mediated cell fusion and examined its relationship to syncytium formation detected by light microscopy.

Several biochemical fusion assays have already been developed using indicator genes whose transcription is activated by cell fusion. Some assays are based on transcriptional transactivation by HIV-1 tat of an indicator gene under the control of the HIV LTR. The HIV-1 tat system does not work well in murine cells because tat transactivation requires species-specific cell factors (Bieniasz et al., 1998). The tat-based cell fusion assay is also subject to nonspecific activation or repression (Barbeau et al., 1997; Berube et al., 1996; Klebanoff et al., 1997). Cell fusion assays have also been based on expression of T7 RNA polymerase in one cell and an indicator gene driven by the T7 promoter in another cell (Barbeau et al., 1998; Nussbaum et al., 1994). Usually, this system involves the use of vaccinia vectors to achieve high levels of T7 polymerase in the cytosol and high levels of cytosolic DNA bearing the T7 DNA promoter-driven indicator gene. While extremely sensitive, such systems require safety precautions appropriate for work with vaccinia virus and are restricted to cells that vaccinia can infect.

To avoid these complications, we turned to another potent transcriptional activation system based on a bacterial tetracycline (tet)-regulated promoter adapted for use in mammalian cells (Gossen and Bujard, 1992). Transcription from the tet operator can be strongly induced or strongly inhibited by a tetracycline transactivator protein in a tetracycline-regulatable fashion. This system has been widely used to control gene expression in single cell types. We adapted it to detect cell fusion by putting the transactivator in one cell and the transactivator-controlled reporter gene in another cell. Fusion between cells mediated by several different virus envelope and receptor genes tested led to strong induction of the indicator gene. Using this system, we found that the cell fusion competence of His8 mutants of ecotropic MLV envelope parallels their competence for virus entry, and that virus-mediated cell fusion can be extensive despite being limited mainly to fusion between pairs of cells. It may be useful to consider giant-cell syncytium formation as a progressive form of cell fusion that escapes normal, self-limited processes.

**Results**

**Development of the cell–cell fusion assay based on Tet-Off gene expression system**

Tetracycline transcriptional transactivation systems are commonly used for analysis of gene expression, with the transcriptional transactivator and reporter gene introduced into the same cell (Baron and Bujard, 2000). We adapted it to detect cell fusion as illustrated in Fig. 1. One cell line was engineered to express a fusogenic form of the MLV envelope (Rein et al., 1994) and the tetracycline-controlled transactivator (tTA), a chimeric protein composed of the N-terminal 207 amino acids of the DNA-binding Tet repressor protein (TetR) and the C-terminal 127 amino acids of the Herpes Simplex virus VP16 activation domain (Gossen et al., 1994). Another cell line was engineered to express the ectopic MLV receptor protein mCAT1 (Albritton et al., 1989) and a luciferase reporter under the control of a tetracycline-response element (TRE) located just upstream of a minimal CMV promoter (P\text{min}\text{CMV}) (Gossen and Bujard, 1992). Constitutive expression of luciferase is usually low due to the lack of enhancer elements in P\text{min}\text{CMV} but highly inducible when tTA binds to the TRE. Fusion between the engineered cells induces transcription of luciferase, detection of which in a light-emitting assay provides a quantitative measure of fusion.

In constructing an indicator cell line, it is desirable that the reporter gene be expressed at low levels before induction and high levels afterward. Since basal expression varies in different cell lines (Howe et al., 1995) and integration sites (Paulus et al., 2000), we first compared GFP expression in a variety of cell lines transiently transfected with pTRE2pur-EGFP, a vector encoding enhanced green fluorescent protein (EGFP) under the control of the tTA-inducible promoter (TRE-P\text{min}\text{CMV}). U2OS cells and COS7 cells had low background GFP expression with pTRE2pur-EGFP compared to HEK293, BHK, and XC cells. U2OS cells were chosen for further work, transfected with a related plasmid (pTRE2pur-Luc) in which luciferase replaced EGFP, and selected with puromycin. Individual clones were screened for low background luciferase activity and high induction following transient transfection with a tTA expression vector (pTet-Off). One such clone (U2OS-Luc) was chosen for further work, transfected with a vector (pECAT-GFP) encoding the MLV receptor (mCAT1) fused to EGFP and selected with G418. The mCAT1-GFP fusion gene was used because it was previously shown to be fully functional as a receptor (Lee et al., 1999; Lu and Silver, 2000; Masuda et al., 1999) and it permitted selection of cells expressing high levels of receptor by flow cytometry for bright GFP fluorescence. The resultant indicator cell line was designated U2OS-Luc-CATGFP.

For a fusion partner cell, we chose a previously established BHK cell line that stably expresses fusogenic MLVenv (Kazachkov et al., 2000); these cells were transfected with pTet-Offpur and selected with puromycin, yielding a cell line designated BHKenv-tTA. Co-culture of BHKenv-tTA cells with U2OS-Luc-CATGFP indicator cells led to cell fusion and induction of luciferase to levels 100–1000-fold greater than in controls in which the MLVenv or tTA gene was absent (Fig. 2A). As expected, induction of luciferase was completely suppressed by treatment with 1–5
μg/ml tetracycline during co-cultivation (Fig. 2B), which binds tTA and prevents its binding to TRE (Gossen and Bujard, 1992).

Characterization of the quantitative cell–cell fusion assay

We characterized the fusion assay concerning sensitivity to the number of fusion events, time for induction of luciferase, and reproducibility. When varying numbers of U2OS-Luc-CATGFP, cells were co-cultured with 10^5 BHKenv-tTA cells, the increase in luciferase activity was proportional to the number of U2OS-Luc-CATGFP cells from approximately 10^2 to 10^5 cells (Fig. 2C) and leveled off as the ratio of indicator cells to BHKenv-tTA cells approached 1 (data not shown). Time course studies showed that the increase in luciferase activity could be detected 2–4 h after co-cultivation and peaked around 16 h (Fig. 2D). Repetition of the assay in triplicate on different days indicated high reproducibility (Fig. 2E).

Because miniaturization of the assay would be potentially useful for screening drugs or treatments that modulate fusion, we investigated a microtiter-well format with 2 × 10^4 indicator and 2 × 10^4 BHKenv-tTA cells per well; cell fusion resulted in an approximately 300-fold increase in luciferase activity and the variability between wells was small (Fig. 3A).

For other applications, transient transfection of fusion-inducing genes would be useful. We transiently transfected MLVenv and/or tTA genes into HEK293 cells and co-cultured them with U2OS-Luc cells transiently transfected with the ecotropic MLV receptor. Luciferase activity increased over 100-fold following co-culture. As expected, MLVenv, tTA, and the ecotropic MLV receptor were all required for increased luciferase activity (Fig. 3B).

To see if the assay would work with other viral fusion proteins, we co-cultured indicator cells with HEK293 cells transiently transfected with the tTA expression vector plus expression vectors for amphotropic MLV envelope, HIV-1 envelope, or VSV-G (Clontech, Palo Alto, CA). Luciferase activity increased approximately 300-fold when we transfected amphotropic MLV envelope lacking its cytoplasmic tail (Fig. 3B). No increase in luciferase activity was detected when vectors encoding the full-length amphotropic or ecotropic envelope was used (data not shown). This shows that the cell-fusion assay—like virus entry—requires the “fusion-activated” form of MLV envelope with a
truncated cytoplasmic tail (Rein et al., 1994). For experiments with HIV-1 envelope, we used a human glioma cell line (U373-MAGI-CXCR4-CEM) engineered to express the HIV receptors CD4 and CXCR4 (Vodicka et al., 1997). An approximately 700-fold increase in luciferase activity was observed when these cells were transiently transfected with the luciferase reporter and co-cultivated with HEK293 cells transfected with pTet-Off plus an expression vector for a CXCR4-using HIV-1 envelope (Fig. 3B). Control experiments in which the HEK293 cells were transfected with pTet-Off plus a CCR5-using HIV-1 envelope (AD8) did not result in increased luciferase activity, although the CCR5-using envelope-transfected HEK293 cells did fuse with a CEM.NKR-CCR5-Luc indicator cell line bearing CD4 and CCR5 (Spenlehauer et al., 2001; Trkola et al., 1999), showing that the cell fusion assay requires the correct coreceptor in the case of HIV-1. Luciferase activity increased approximately 100-fold when pVSV-G was used as the fusogenic envelope. Since VSV-G fusion is enhanced by low pH, we treated co-cultured cells briefly with pH 5.5 medium; this massively increased syncytium formation but did not increase luciferase activity (Fig. 3C). In contrast to the results with ecotropic MLV, the mCAT1 receptor was unnecessary for fusion mediated by VSV-G (Fig. 3C) or amphotropic MLV env (not shown). This is expected because functional receptors for VSV-G and amphotropic MLV, but not ecotropic MLV, are expressed on human cells.

Complementation of MLV envelope histidine 8 mutant-mediated cell fusion by soluble receptor-binding domain (RBD)

Histidine at position 8 in the ecotropic MLV envelope is not close to the receptor-binding domain in the crystal structure of MLV envelope (Fass et al., 1997), and viruses in which histidine 8 is deleted or replaced with arginine or alanine bind receptor normally but are not infectious (Bae et al., 1997). Interestingly, the block to virus infection with these mutants can be reversed by incubating cells with a soluble form of the amino-terminus of the mature envelope, which contains the receptor-binding domain (RBD) (Barnett and Cunningham, 2001; Barnett et al., 2001). Co-culture of U2OS indicator cells with HEK293 cells trans-
fected with pTet-Off plus H8A or H8R versions of MLV envelope showed no increase in luciferase activity, but addition of approximately 4 nM RBD peptide resulted in a 50-fold increase in luciferase activity (Fig. 4). This shows that the cell fusion competence of His8 mutants of ecotropic MLV envelope parallels their competence for virus entry. The same concentration of RBD mildly inhibited the fusion activity of cells expressing wild-type ecotropic envelope, consistent with competition between soluble RBD and cell-membrane expressed envelope for receptor binding sites.

**Significant fusion without obvious syncytium formation**

Although the cytoplasmic tail-truncated version of MLV envelope induces syncytia in some cell types, we did not observe syncytia above background levels in U2OS-Luc-CATGFP cells cultured with cells expressing fusogenic

Fig. 4. HEK293 cells (2 × 10^5) transfected (or not) with pTet-Off (tTA) and the Mo-MLV envelope expression vector pCEETR with wild-type sequence or histidine 8 replaced with alanine (H8A) or arginine (H8R) were cultured overnight with an equal number of U2OS-Luc-CATGFP cells in the presence of the indicated concentration of RBD and assayed for luciferase activity the next morning. Mean of duplicate assays is shown; range was <10% of mean.
MLV envelopes (Figs. 5A,B), despite greater than a hundred-fold induction of luciferase activity. We considered this surprising because induction of luciferase must involve transfer of tTA from envelope-expressing cells to indicator cells. To look more carefully for evidence of cell fusion, we labeled the MLV-env-expressing HEK293 cells by co-transfecting an expression vector for a mitochondrially targeted version of the DsRed fluorescent protein (Ou and Silver, 2004).

Fig. 5. HEK293 cells transfected with the ecotropic MLV env expression vector pCEETR (A, C) or not transfected (B) were co-cultured overnight with U2OS-Luc-CATGFP cells (A, B) or XC cells (C). Numerous syncytia are seen in C but not in A or B.

Fig. 6. U2OS-Luc-CATGFP cells were cultured in the presence of approximately 5 nM RBD with HEK293 cells that had been transfected with expression vectors for mitochondrially targeted DsRed (pDsRed-Mito) plus ecotropic MLV envelope bearing the H8A mutation. (A) DIC image. (B) Overlay of red and green fluorescence images from the same field shown in A or a different field (C). Fused cells indicated with arrows. Cells expressing CATGFP alone or DsRed-Mito alone are labeled c and m, respectively, in panel A. (D) DIC image of background U2OS-Luc-CATGFP syncytium in same culture. (E) Fluorescence overlay of the same field shown in D. (F) Background syncytium in pure culture of U2OS-Luc-CATGFP cells.
These cells were co-cultured with U2OS-LucCATGFP indicator cells, in which the GFP-labeled MLV receptor is located mainly on the plasma membrane. Confocal microscopy revealed the presence of binucleate cells with red fluorescent mitochondria and green fluorescent plasma membrane, indicating cell fusion, when wild-type envelope was expressed (not shown), or when H8A envelope was expressed in the presence of RBD (Figs. 6A–C). While slightly larger than cells containing single fluorescent labels, the doubly labeled cells were not identifiable as syncytia when observed with transmitted light (Fig. 6A). Thus, cell fusion does occur in cultures showing induction of luciferase, but it can be subtle and not proceed to formation of multinucleate giant cells of the type seen in co-cultures with highly fusogenic XC cells (Fig. 5C). U2OS cultures—like many other cell lines—also contain spontaneous multinucleate giant cells, identifiable as syncytia without red mitochondria in our assay (Figs. 6D–F); such cells complicate attempts to quantify virus-mediated cell fusion by counting syncytia.

Discussion

The quantitative cell fusion assay described here has several advantages compared to existing cell fusion assays. The transcriptional activation is based on a well-characterized, highly controlled, commercially available expression vector system. Because it avoids the use of virus vectors, it is safer and more convenient than assays that require vaccinia or other viruses. Unlike transactivation mediated by the HIV tat protein, which does not work well in cells from certain species due to the need for species-specific cellular co-factors (Bieniasz et al., 1998), the tetracycline transcriptional transactivator is not species-restricted. While we found that the tet-transactivator system did not work well in some cell types due to a high background of luciferase expression in the absence of tTA, newer versions of the luc indicator plasmid with tighter regulation (pTRE-tight-Luc, Clontech) have been developed that may mitigate this problem (http://wwwbdbiosciences.com/clontech/products/literature/pdf/brochures/Tet-On_Tet-Off.pdf).

The assay we describe easily detected fusion with the four virus envelopes tested: ecotropic MLV (which uses the mCAT1 receptor), amphotropic MLV (which uses Pit-2), VSV, and HIV-1. Obvious modifications should allow the assay to be used with other viruses or fusogenic cell proteins. The assay is sensitive enough to work in microtiter format and following transient transfection of fusion genes; hence, it may be useful to screen agents that inhibit fusion, or to search libraries for genes that promote fusion. Numerous controls in our experiments showed that transfer of tTA across cell types is dependent on expression of fusion-competent envelope and receptor genes.

The assay might also be useful to detect cell fusion occurring in vivo. Recent studies show that stem cell differentiation may result from fusion between stem cells and differentiated cells of the host (Terada et al., 2002; Ying et al., 2002). Transgenic mouse strains have been created that carry tTA-activated GFP and lacZ indicator genes (Krestel et al., 2001), while other strains carry tTA transgenes (Kistner et al., 1996). If the background of GFP and lacZ is low enough, it might be possible to detect rare fusion events between stem cells from tTA-expressing strains and host cells in tTA-controlled reporter mice by virtue of enhanced GFP or lacZ in individual fused cells. Along the same lines, the assay might be used to study agents that promote cell fusion in such mice, which could be useful for stem cell therapy.

The main advantage of the assay is that it provides a simple, objective, quantitative measure of envelope-mediated cell fusion. In the case of MLV, most (but not all; see Yang and Compans, 1997) previous assays have relied on light-microscopic quantitation of syncytium formation, which is time-consuming, limited to certain cell types, affected by envelope sequence in ways that do not correlate with viral infectivity, and complicated by background syncytia and subjectivity in selecting which fields to count. We found poor correlation between syncytia detected by routine light microscopy and increased luciferase activity in the cell fusion assay. This is most likely because syncytia too small to be detected by routine microscopy were responsible for enhanced luciferase production in some cell combinations. While we cannot rule out “kiss and run” transient cell fusion (Valtorta et al., 2001) or transfer of envelope-coated microvesicles (Schartz et al., 2002) containing tTA as a cause of transcriptional transactivation, such events would likely transfer less tTA and therefore be much less potent in inducing luciferase than full cell fusion. They would, however, be consistent with luciferase induction in the absence of syncytia. Limitation of cell fusion to pairs of cells as we observed could have many causes, including low levels of envelope or receptor on the cell surface, reduction in concentration of fusion proteins on the cell surface after fusion takes place, or other post-fusion membrane changes that inhibit subsequent fusion events. We favor the second hypothesis because envelope and receptor molecules are known to interact intracellularly when expressed in the same cell, altering post-translational modification and trafficking (Kim and Cunningham, 1993; Wang et al., 1996). Post-fusion intracellular interaction leading to down-regulation of envelope or receptor could provide a natural mechanism to limit subsequent fusion. The availability of functional, fluorescent-protein-labeled MLV envelope (Kayman et al., 1999), and receptor proteins in living cells may allow new approaches to visualizing these proteins during and after cell fusion in cells in which fusion is limited or progressive. Restricting cell fusion to pairs of cells would likely promote the viability of heterokaryons and could have practical applications in stem cell therapy in view of the reports that cell fusion is responsible for some forms of stem cell transdifferentiation.
Methods

Plasmids

pTRE2pur-EGFP was constructed by inserting the EGFP gene, cut from pEGFP-N1 (Clontech) with BamHI/NorI, into pTRE2pur (Clontech) cut with the same enzymes. pECAT-GFP was made by inserting mCAT1-GFP, cut from pSINrep19CAT1GFP (Kazachkov et al., 2000) with BamHI/NorI, into pEGFP-N1 cut with BglII/NorI. To construct pTet-Offpur, the neomycin gene in pTet-Off (Clontech) was excised with XhoI and replaced with the puromycin gene cut from pTRE2pur with the same restriction enzyme. Both pTet-Offpur and pTet-Off encode the tTA. Vectors for expressing ectropic and amphotropic MLV envelopes with full-length or truncated cytoplasmic tails (pCEE, pCEETR, pCAE, and pCAETR) were kind gifts from Jack Ragheb (Ragheb and Anderson, 1994). Additional vectors encoding Mo-MLV envelope were made by replacing GFP in pEGFP with segments of Mo-MLV envelope made by PCR; these vectors gave equivalent results as pCEE and pCEETR. Mutations at amino acid 8 in Mo-MLV were made by PCR mutagenesis and confirmed by sequencing. The RBD of Mo-MLV was prepared generally following a previously published method (Davey et al., 1997). A cDNA encoding RBD-His was prepared following a previously published method (Davey et al., 1997). A cDNA encoding the N-terminal 268 residues of Moloney Murine Leukemia virus envelope (receptor binding domain, RBD) with a C-terminal protease cleavage site and His6 tag was generated by polymerase chain reaction using RBD-forward primer 5′AGTACTAGTACTGACATTGGCGGCGTCAAC3′, RBD- His reverse primer 5′GGTACTCGACTAGTGTGATGGTGATGTGATGGCCCTGAAAATACAGGTTTTCGTCGTCGGGATATCGTAATCTATTGGGACGCGGGTG-ATGATGGTGATGGCCCTGAAAATACAGGTTTTGATCGTCGTCGGGATATCGTAATCTATTGGGACGCGG, and a shorter version of the reverse primer 5′GGTACTCGACTAGTGTGATGGTGATGTGATGGCCCTGAAAATACAGGTTTTGATCGTCGTCGGGATATCGTAATCTATTGGGACGCGGGTG-ATGATGGTGATGGCCCTGAAAATACAGGTTTTCGTCGTCGGGATATCGTAATCTATTGGGACGCGGGTG-ATGATGGTGATGGCCCTGAAAATACAGGTTTTGATCGTCGTCGGGATATCGTAATCTATTGGGACGCGG, and inserted into XhoI, SpeI, and a shorter version of the reverse primer 5′GGTACTCGACTAGTGTGATGGTGATGTGATGGCCCTGAAAATACAGGTTTTGATCGTCGTCGGGATATCGTAATCTATTGGGACGCGGGTG-ATGATGGTGATGGCCCTGAAAATACAGGTTTTCGTCGTCGGGATATCGTAATCTATTGGGACGCGG. The PCR product was digested by SpeI and inserted into SpeI site of pFastBac1 vector (Invitrogen, Carlsbad, CA). One nanogram of pFastBac1-RBD-His6 was transformed into DH10BAC competent cells (Invitrogen) to produce recombinant bacmid DNA. Bacmid DNA was transfected into S9 cells to produce recombinant virus, which was amplified by infection of S9 cells. For production of RBD, S9 cells were grown to 2 × 10^6 per milliliter in suspension at 27 °C with shaking and infected with recombinant baculovirus at a multiplicity of infection of 5. Two days later, supernatants were collected by centrifugation for 5 min at 500 × g, incubated for 1 h in a cold room with ProBond resin (Invitrogen, 20 ml of resin per liter of medium). The resin was loaded on a column by gravity, and washed with 10 column volumes each of 20 mM sodium phosphate–500 mM NaCl (pH 6.0) and 50 mM imidazole–20 mM sodium phosphate–500 mM NaCl (pH 6.0). The bound protein was eluted with 500 mM imidazole–20 mM sodium phosphate–500 mM NaCl (pH 6.0) and dialyzed against 500 mM NaCl–20 mM HEPES (pH 7.4) for 2 days. Protein concentration (205 μg/ml, ~7 μM) was determined with BCA kit (Pierce). Flow cytometry indicated that the purified RBD (detected with antibody to the His tag) bound to cells expressing mCAT1 (XC, NIH3T3, HEK293-CATGFP) but not to control cells (HEK293, MLV-infected NIH3T3 cells).

Transfection and selection

Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 U of penicillin per milliliter, 200 μg of streptomycin sulfate per milliliter, and 2 μM L-glutamine (DMEM+) at 37 °C in a 5% CO2 incubator. For transfection, 10^6 cells were seeded per well of a six-well plate, cultured at 37 °C overnight, and transfected by replacing the medium with 0.5 ml of serum-free Opti-MEM medium (Gibco, Frederick, MD) to which 2–4 μg DNA and 4–8 μl LipofectAMINE2000 (Invitrogen) were mixed per the manufacturer’s instructions. After 3–6 h at 37 °C, the medium was replaced with DMEM+ medium. To select stable cell lines, transfected cells were split 24 h after transfection and incubated with DMEM+, containing 1 mg/ml G418 or 4 μg/ml puromycin. Medium with neomycin or puromycin was changed every 72 h for 2 weeks. Cells expressing high levels of mCAT1-GFP were selected by flow cytometry for high GFP fluorescence with a FACStar Plus cell sorter (BD, Franklin Lakes, NJ).

Cell fusion assay

Stably or transiently transfected target and indicator cells were trypsinized, mixed at different ratios, plated in replicate wells, and incubated at 37 °C in a 5% CO2 incubator for 16 h (or other times as indicated). Luciferase activity was quantified with the Luciferase Assay System (Promega, Madison, WI). Briefly, culture medium was removed and cells were lysed with 0.2–0.5 ml of passive lysis buffer in which they were frozen on dry ice for 5 min, thawed in a 37 °C water bath, and vortexed. After spinning in a microcentrifuge at 12000 rpm for 5 min to remove cell debris, 100 μl of luciferase assay reagent was injected into 20 μl of supernatant and the luminosity was measured with an Opto-comp II luminometer (MGM Instruments, Hamden, CT).

Confocal microscopy

Cells were grown in LabTek Chambered Coverglass devices and examined with a Leica SP2-AOBS confocal microscope with a 63× oil immersion NA 1.32 objective. GFP and DsRed were excited with argon and krypton lasers at 488 and 568 nm, respectively; detector slits were adjusted to minimize cross-talk between the channels. Images were collected simultaneously using a transmitted light detector. Images were processed using Leica TCS-NT/SP software.
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References


