



VIRAL TRANSFER TECHNOLOGY

RESEARCH ARTICLE

Subcellular post-transcriptional targeting: delivery of an intracellular protein to the extracellular leaflet of the plasma membrane using a glycosyl-phosphatidyl inositol (GPI) membrane anchor in neurons and polarised epithelial cells

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The effectiveness of viral vector-mediated gene transfer depends on the expression of therapeutic transgenes in the correct target cell types. So far, however, little attention has been given to targeted subcellular distribution of expressed transgenes. Targeting individual transgenes to particular subcellular compartments will provide various advantages in increasing the safety, efficacy, and specificity of viral vector-mediated gene delivery. Viruses normally hijack the cellular protein synthesis machinery for their own advantages. It is thus unknown whether cells infected with viral vectors will be able to target proteins to the correct subcellular organelles, or whether the subcellular targeting machinery would be selectively disrupted by viral infection. In this article we explored whether a herpes simplex virus type 1-derived vector could be used to deliver a transgene engineered to be targeted to the extracellular membrane of target cells. To

do so we constructed a temperature-sensitive mutant HSV-1 vector, tsK-TT21 expressing a recombinant marker protein, tissue inhibitor of metalloproteinases (TIMP), linked to sequence encoding a signal for the addition of a glycosyl-phosphatidylinositol (GPI)-anchor within the endoplasmic reticulum. Our results demonstrate that HSV1-derived viral vectors can be used to target transgenes as GPI anchored proteins to the outside leaflet of plasma membranes, without disrupting the targeting machinery of host epithelial cells or neurons. This approach could then be used to target specific proteins to the cell membrane to modify cell–cell interactions, the function of specific plasma membrane proteins, or their interactions with other membrane proteins, and also to target a prodrug converting enzyme to the plasma membrane of target cells, therefore enhancing its cell killing effects. Gene Therapy (2000) 7, 1947–1953.

Keywords: viral vectors; targeting; herpes simplex vectors; neuronal polarity

Introduction

The capacity to display a therapeutic protein specifically on the outside of target cells could have many advantages in the design of gene therapy approaches. In particular, it could be designed to modify cell–cell interactions, the function of plasma membrane proteins or their interactions with other membrane proteins. It could also be used to target a prodrug converting enzyme to the plasma membrane of target cells, therefore enhancing the bystander effect.

Marais *et al*¹ highlighted the benefits of expressing the bacterial enzyme carboxypeptidase G2 (CPG2) as a transmembrane protein for use in gene-directed enzyme prodrug therapy (GDEPT). This retargeting was achieved by the fusion of the transmembrane region of the human

tyrosine kinase receptor c-erb B2 to the C-terminus of CPG2. Stable expression of this chimaeric protein on the outer membrane of the breast carcinoma cell line MDA MB 361 rendered these tumour cells sensitive to the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA) which cannot cross the plasma membrane. Cells expressing membrane-bound CPG2 were able to allow the extracellular conversion of CMDA into its non-polar cytotoxic metabolite (which can thus cross the plasma membrane), and thus elicit an important bystander effect following prodrug administration.¹ This strategy would pave the way for new enzyme prodrug combinations where potential prodrugs are unable to cross the plasma membrane of cells and could improve existing GDEPT systems that require cell to cell communication to give a bystander effect such as thymidine kinase/ganciclovir.^{2–4}

Cell surface display of proteins is also being investigated as a way of improving existing immunotherapy strategies for cancer gene therapy. The surface expression of an exogenous protein *per se* may be used to stimulate in specific ways the cell–cell interactions needed to stimu-

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late adequate antitumoral humoral and cellular immune responses.⁵ As an example of such a strategy, the costimulatory molecules B7-1 and B7-2 were engineered to encode for the GPI anchoring signal of the decay accelerating factor, and have been expressed in stably transfected EG7 tumour cells. Membrane preparations from these cells were used to immunize mice with the induction of tumour-specific T cell proliferation and a cytotoxic T lymphocyte response.⁶ The targeting of plasma membrane proteins via glycolipid anchors, encoded within viral vectors, however, has not yet been attempted. It is thus not known, whether such chimaeric proteins would be targeted to the correct compartment, or whether the viral vector would disrupt the intracellular protein targeting machinery. If feasible, this approach will allow the targeting of transgenic proteins exclusively to the apical domain of polarised epithelial cells, eg the intraluminal membrane of intestinal epithelial cells.

Efficient *in vivo* delivery of these therapeutic genes is fundamental to gene therapy. Viral vectors are widely used to achieve highly efficient transfer of the transgene but until now the ability of these vectors to deliver a protein specifically to the apical membrane of a polarised cell has not been examined. We have previously described the temperature-sensitive mutant HSV-1 vector *tsK-TT21*, which cannot replicate at 37°C or higher, and encodes a fusion gene consisting of human tissue inhibitor of metalloproteinases (TIMP), linked to the last exon of Thy1.⁷ TIMP is a naturally secreted protein normally not expressed in neurons. We have now used *tsK-TT21* to investigate whether viral vector delivery of this GPI-modified marker protein will disrupt the targeting machinery of the host cell or result in the successful retargeting of TIMP to the outer plasma membrane. Furthermore, the selective targeting of transgenic proteins to either the neuronal dendrites or axons, will also be important to specifically engineer the function of neurons *in vivo*.

Polarised epithelial cells (Madin-Darby canine kidney – MDCK) and polarised neocortical neurons were infected with *tsK-TT21* to investigate the effect of virus infection on both the polarity of these cells as well as the polarised distribution and GPI anchoring of TIMP/Thy1 using biochemical and immunocytochemical methods.

Importantly, the HSV-1 vector did not disrupt the polarity of either infected epithelia or neurons. TIMP/Thy1 was recognised as a GPI anchored membrane protein and correctly processed as such in both cell types. MDCK cells expressed the TIMP protein exclusively on their apical plasma membrane following the strict sorting pattern of GPI anchored proteins observed in these cells. Viral delivery of the TIMP/Thy1 into neuronal cells unexpectedly resulted in its expression being predominantly localised to the somato-dendritic membrane. Our results therefore show that a viral vector can be exploited to deliver a transgene which is to be expressed on the extracellular leaflet of the apical membrane of fully polarised epithelial and fully polarised neuronal cells.

Results

Viral infection does not disrupt the polarity of MDCK cells

To assess whether virus infection *per se* may alter cellular polarity in a nonspecific manner, which in turn may

affect plasma membrane targeting, we investigated the effect of *tsK-TT21* infection upon the polarity of MDCK cells. Integrity of the polarity of the MDCK monolayer was assessed by measuring the rate at which the radiolabelled macromolecule ³H inulin (M_r 5000) could pass from the apical compartment to the basolateral compartment of filter grown MDCK cells (Figure 1). ³H inulin was added at $t = 0$. At the indicated times both apical and basolateral medium was sampled and the percentage of basolateral/apical c.p.m. (counts per minute) calculated. Physical mechanical disruption of the monolayer was achieved by a needle scratch, and loss of the polarized distribution of ³H inulin was immediate. Viral infection with *tsK-TT21* at the permissive temperature of 31°C to allow viral replication disrupted the membrane from 4 h onwards. Following infection at the nonpermissive temperature for viral replication of 37°C, viral infection had no effect on the maintenance of epithelial polarity, demonstrating that the MDCK cells remained fully polarised after infection with nonreplicating virus with continued transgene expression.

TIMP-Thy1 is appropriately sorted to the apical membrane of fully polarised epithelial cells

To confirm that virally expressed TIMP/Thy1 was correctly sorted to the outer plasma membrane of polarised epithelial cells we expressed TIMP/Thy1 in polarised MDCK cells. Polarised MDCK cells strictly sort both exogenous and endogenous GPI-anchored proteins to their apical membrane. MDCK cells were grown on filters to allow the cells to become completely polarised. Twelve hours after infection with *tsK-TT21* distribution of

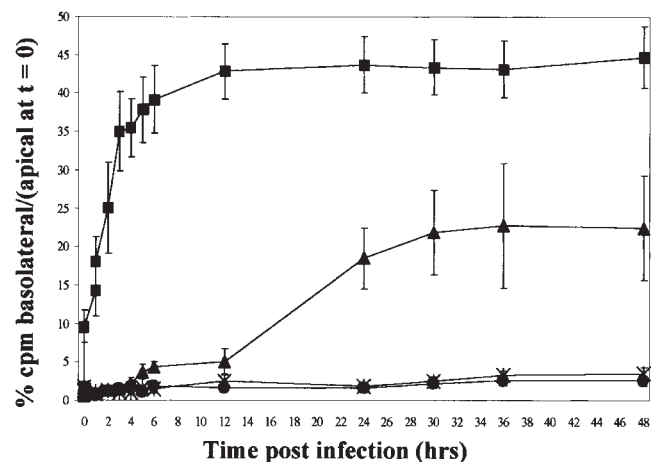


Figure 1 Viral infection does not disrupt monolayer permeability of polarised MDCK cells. Integrity of the MDCK monolayer, following infection with *tsK-TT21*, was assessed by measuring the rate at which the radiolabelled macromolecule ³H inulin (M_r 5000) could pass from the apical compartment to the basolateral compartment of MDCK cells grown on filters. ³H inulin was added at $t = 0$, then at the indicated times both apical and basolateral medium was sampled and the percentage of counts per minute (c.p.m.) calculated as basolateral c.p.m./apical c.p.m. Immediate physical disruption of the monolayer was achieved by a needle scratch (■, positive control). The monolayer remained intact following the addition of 3 μ l media and after 48 h only 3% of the added inulin had traversed into the basolateral compartment (●, negative control). Viral infection with *tsK-TT21* at the permissive temperature of 31°C to allow viral replication, disrupted the membrane by 5% after 4 h (▲) and by 18% following 24 h of infection. At the nonpermissive temperature of 37°C viral infection had no effect above that observed in the negative control (×).

TIMP/Thy1 was analysed (Figure 2). The basolateral plasma membrane domain was selectively labelled by biotinylation of its extracellular surface proteins. TIMP/Thy1 was targeted to the apical membrane of polarised MDCK cells producing a distinct punctate membrane pattern following TIMP immunocytochemical detection, similar to that obtained for other apically targeted proteins. Neither the protein nor RNA synthesis inhibitors, cycloheximide or actinomycin D respectively, used to treat the cells 12 h after infection, altered the pattern of staining, demonstrating that TIMP-Thy1 is stably expressed at the plasma membrane and ruling out the possibility that membrane staining was only transient, due to its secretion from cells, intracellular degradation, or its redistribution to a different subcellular compartment. This confirmed that the GPI anchor of TIMP/Thy1 was being recognised and used as an apical targeting signal in epithelial MDCK cells even when a viral vector was used for the delivery of this marker protein.

HSV-1 encoded TIMP-Thy1 is targeted to the somato-dendritic domain of polarised neocortical neurons

We used *tsK-TT21* to express TIMP/Thy1 in 10-day-old, fully polarised neocortical neurons in low density pri-

mary cultures, infecting neurons at 37°C, the nonpermissive temperature for replication. Fourteen hours after infection neurons were analysed (Figure 3). The somato-dendritic and axonal domains of polarised neurons were clearly differentiated using two cytoskeletal protein markers, the dendritic marker Map 2 (Figure 3, panels b and d), and the axonal marker tau (Figure 3, panels a and g). Immunostaining for TIMP (Figure 3, panels e and h), and its comparison with the distribution of the dendritic and axonal markers, demonstrates that the majority of immunoreactive TIMP is localised to the cell bodies and dendrites. Therefore, this GPI-anchored marker protein is localised to the somato-dendritic plasma membrane domain of neocortical neurons.

TIMP/Thy1 is GPI localised to the extracellular leaflet of the plasma membrane of both polarised MDCK cells and neocortical neurons

Having confirmed that TIMP/Thy1 is sorted in a polarised manner when virally delivered to both epithelia and neurons we further examined whether the TIMP/Thy1 was being correctly translated and attached to the extracellular surface of both MDCK and neocortical neurons via a GPI anchor. We identified extracellular proteins by

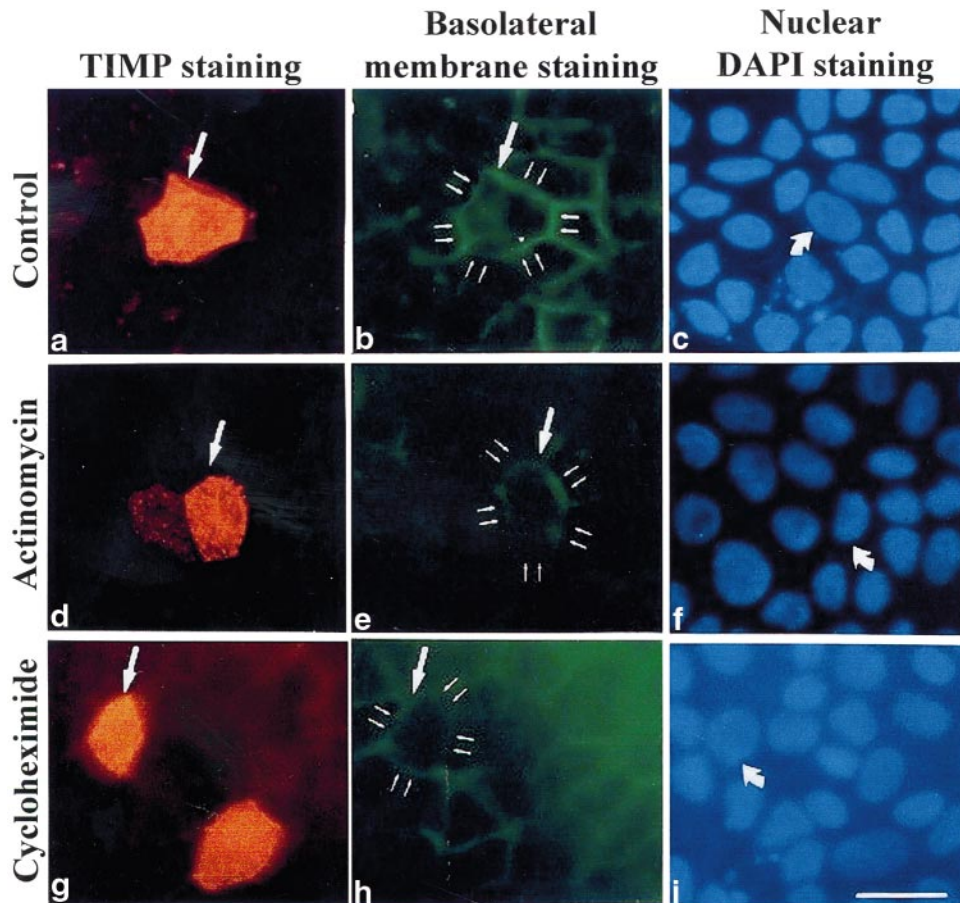


Figure 2 Exogenous GPI-anchored proteins are appropriately sorted to the apical membrane of polarised MDCK epithelial cells when expressed from a viral vector. Expression of TIMP/Thy1 in polarised MDCK cells infected with *tsK-TT21* at MOI 10, studied 12 h after infection following fixation and immunostaining for TIMP and biotinylated surface proteins. Rhodamine (red) staining shows apical TIMP/Thy1 expression (panels a, d and g). Fluorescein staining (green) identifies basolateral biotinylated proteins (panels b, e and h) with the small white arrows outlining polarised infected cells. The third group (panels c, f and i) shows nuclear staining with the large white arrows again denoting infected cells expressing TIMP/Thy1. The cells in panel group 2 were treated with cycloheximide and those in panel group 3 with actinomycin D, neither of which altered the distribution of either the basolateral or apical markers. Scale bar (shown in bottom right panel) = 20 μ m.

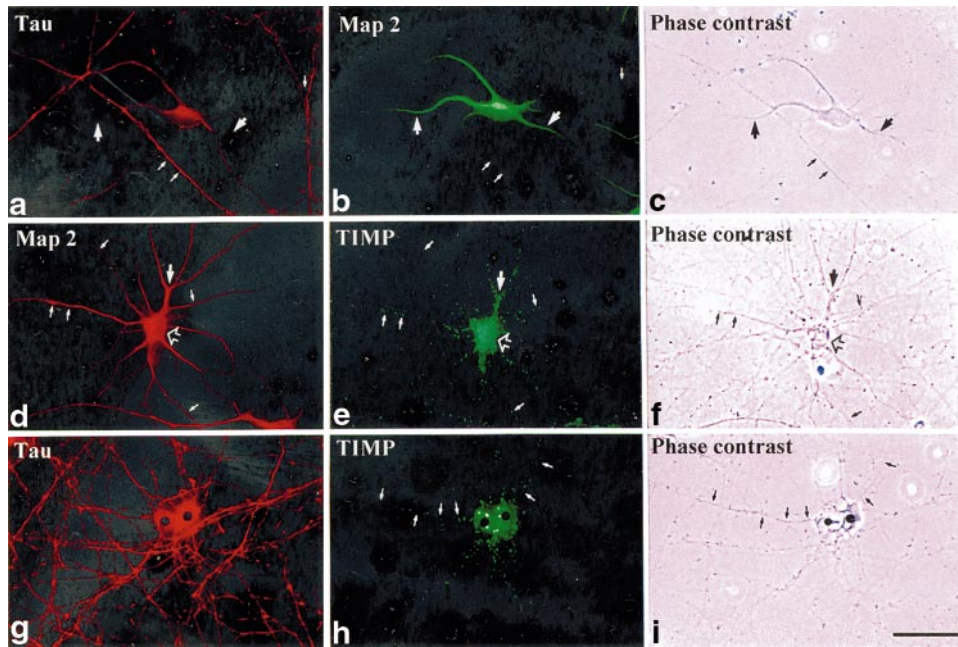


Figure 3 Viral vector delivery of the exogenous GPI-anchored protein, TIMP/Thy1 to polarised post-mitotic neocortical neurons results in its localisation to the somato-dendritic plasma membrane domain. Expression of TIMP/Thy1 in 10-day-old neocortical neuronal cultures infected with tsK-TT21 at an MOI 10 and studied 14 h after infection, following fixation, permeabilisation and immunostaining with anti-Map 2 (b and d), anti-tau (a and g) and anti-TIMP antibodies (e and h). Panels c, f and i show the cultures under phase contrast. Map 2 staining is localised to the cell bodies and dendrites (open white arrows indicate the location of the cell bodies and larger white arrows identify dendrites), while tau staining is restricted to axonal processes (small white arrows). Identical arrows are shown for each row. Having clearly identified the somato-dendritic and axonal membrane domains using these two cytoskeletal protein markers, panels e and h show that the majority of TIMP is in fact localised to the cell bodies and dendrites. Scale bar (shown in i) = 20 μm .

biotin labelling *in vitro*, allowing us to recognise the 25 kDa TIMP/Thy1 protein by immunostaining membrane preparations, extracted into the detergent phase using Triton X-114 extraction, for either biotin or TIMP. In both MDCK cells and neocortical neurons TIMP/Thy1 had been biotinylated and extracted into the detergent phase revealing that it was expressed as an extracellular membrane protein in both cell types (Figure 4). GPI anchored proteins can be cleaved off a membrane using phosphatidylinositol-specific phospholipase C (PI-PLC). After cleavage such proteins undergo a conversion from a hydrophobic to a hydrophilic state, thus being released into the cell supernatant. This procedure exposes an antigenic epitope recognised using a monoclonal antibody against the cross-reacting determinant (CRD) of the GPI anchor which remains attached to the released protein following treatment with PI-PLC. To identify TIMP/Thy1 as a GPI-anchored protein, infected MDCK and neocortical neurons were labelled by both immunostaining for TIMP and the CRD epitope (Figure 5). Without PI-PLC cleavage the protein remained on the membrane, therefore in the cell pellet, and was only released into the supernatant after PI-PLC treatment, confirming that TIMP/Thy1 shows the expected behaviour of a GPI-anchored protein.

Discussion

Many cells display various plasma membrane domains. Polarised epithelial cells display two different membrane compartments, the apical and basolateral membrane domains, which are separated by tight junctions. Neu-

ronal cells also display two distinct cellular processes, axons and dendrites, with the accompanying two separate plasma membrane domains, namely the dendritic and the axonal plasma membranes. The function of tight junctions in separating both domains in epithelial cells, is carried out by the axonal initial segment region of neurons. Plasma membrane domains differ anatomically, biochemically and physiologically. Their different biochemical composition is achieved through the selective targeting of proteins to either domain, through the use of domain-specific targeting signals. One of the strongest targeting sequences is the glycosyl-phosphatidyl inositol (GPI) anchors. Endogenous or even any exogenous GPI-anchored proteins are selectively targeted to the outer leaflet of the plasma membrane of epithelial cells and neurons. Furthermore, GPI-linked proteins are targeted selectively to the apical domain of polarised epithelia. In neurons, whilst also targeted to the plasma membrane, they can be targeted to either the dendritic or axonal plasma membrane, although most immunoreactivity was detected on neuronal dendrites.

In experiments using polarised epithelial cells it has been shown that this glycolipid anchor targets either endogenously linked GPI proteins, or engineered GPI-linked proteins to the apical membrane. Proteins engineered to encode for the signal conveying the addition of a GPI anchor are targeted to the apical plasma membrane of polarised epithelia. This is not only independent of whether the anchor is natural or engineered, but the GPI anchor will also over-ride the targeting signal of a secretory protein, which is normally targeted to secretory vesicles.⁸⁻¹³

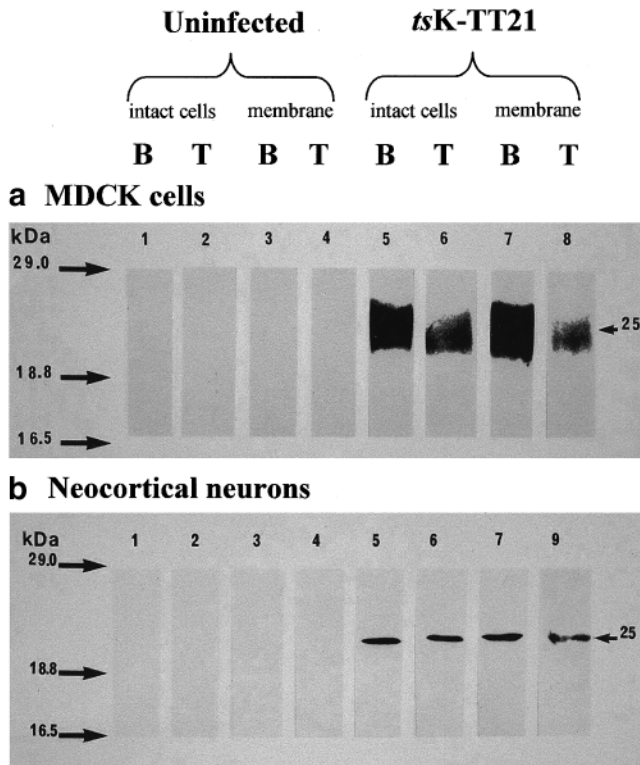


Figure 4 Identification of TIMP-Thy1 as an extracellular membrane protein in cultured MDCK and neocortical neurons. Expression of TIMP/Thy1 in both MDCK and neocortical neurons infected with tsK-TT21 (MOI 10) 14 h after infection. Western blot analysis for MDCK cells (a) and neocortical neurons (b) showing the 25 kDa TIMP/Thy1 protein as indicated by the small black arrows. Lanes 1–4 show uninfected cells and lanes 5–8 infected cells. Neither intact cells (lanes 1 and 2) or detergent extracts (membrane) from uninfected cells (lanes 3 and 4) were immunoreactive for biotin (B), (lanes 1 and 3) or TIMP (T), (lanes 2 and 4). However intact cells (lanes 5 and 6) or detergent extracts (membrane) from infected cells (lanes 7 and 8) were immunoreactive for biotin (lanes 5 and 7) and TIMP (lanes 6 and 8) confirming that TIMP/Thy1 was expressed as a GPI-linked protein (25 kDa) in both cell types, because it partitioned into the detergent phase.

Viral vectors are powerful delivery vehicles for many gene therapy strategies.¹⁴ However, viruses are known to modify the cellular protein synthesis machinery, or the antigen processing capacity of infected cells.¹⁵ The effects of virus infection upon the protein targeting machinery of host cells has not yet been assessed. This is of particular importance if the gene therapy strategy requires that the transgene be expressed in a particular subcellular compartment, eg as an extracellular protein linked to the plasma membrane. Cell surface display of a therapeutic protein would be desirable for vaccine development and human immunotherapy requiring an immune stimulatory/inhibitory molecule to be exposed to the extracellular medium. The capacity to use subcellular targeting signals to target a protein to either the basolateral or apical membrane domain, could also be of use in manipulating specific types of intercellular interactions. It would also allow selective modification of different aspects of neuronal function by either targeting a protein to dendrites, where neurons normally display their post-synaptic receptor proteins, or to the axons, where the neurotransmitter secretory machinery is located; also, in cancer gene therapy strategies such as gene-directed

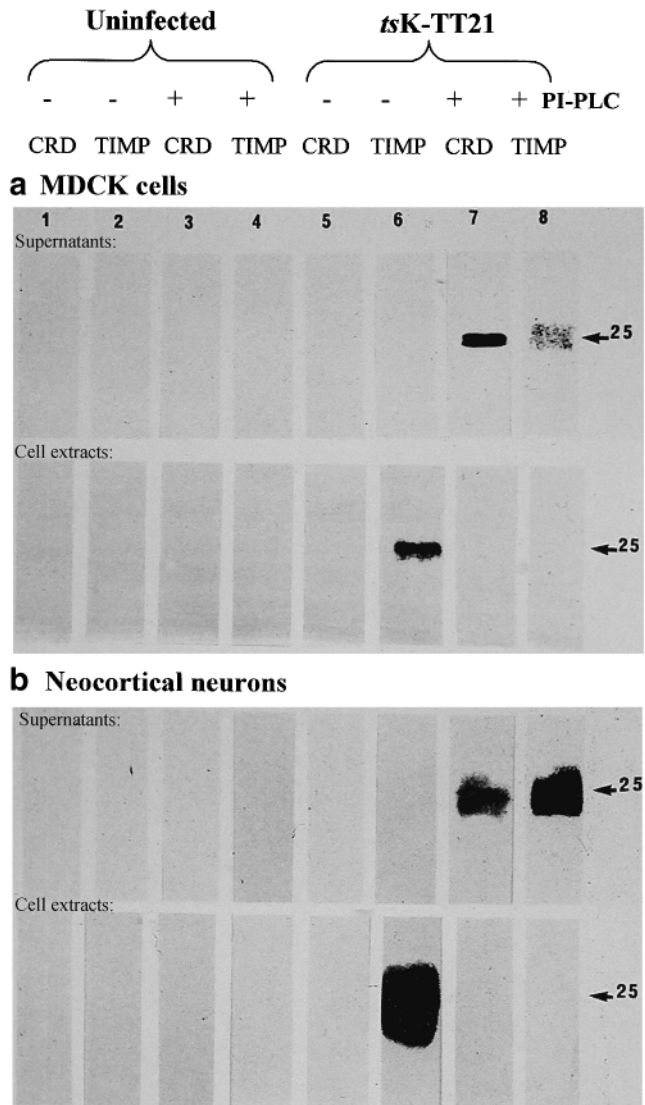


Figure 5 Identification of TIMP/Thy1 as a GPI-anchored protein in cultured MDCK and neocortical neurons. Western blot analysis of both the cell extracts and cell supernatants from MDCK (a) and neocortical neurons (b). The cells were infected with tsK-TT21 (MOI 10) and 14 h after infection the immunostaining pattern for TIMP and the CRD epitope was assessed in both fractions. Without PI-PLC treatment the protein remains in the cellular fraction with the CRD epitope unexposed, therefore a 25 kDa band is only observed when the cell pellet is immunostained for TIMP (cell extract – lane 6). After PI-PLC treatment TIMP is released into the supernatant exposing the CRD epitope producing a 25 kDa band upon immunostaining of the cell supernatants for both the CRD epitope (supernatant – lane 7) and for TIMP (supernatant – lane 8), indicating that TIMP was effectively linked to the membrane via a PI-PLC cleavable GPI anchor.

enzyme prodrug therapy, in which a class of compound highly suitable as a prodrug cannot permeate into cells therefore requiring conversion to a cytotoxic drug by an extracellular enzyme.

Our experiments demonstrate that a viral vector can successfully target a recombinant marker protein to the extracellular leaflet of the plasma membrane of both polarised epithelia or neurons. In addition, the infection with the HSV1-derived vector did not deleteriously affect biochemical, morphological or physiological indices of

the polarity of the infected polarised MDCK cells or polarised neurons,^{16,17} nor the strict plasma membrane sorting mechanisms that exist for GPI-anchored proteins.

Viral vector infection at the nonpermissive temperature for virus replication did not alter the polarity of polarised MDCK cells and TIMP-Thy1 was targeted to the apical domain of these cells. The chimaeric protein TIMP-Thy1 was correctly targeted to the plasma membrane of cultured neurons and detailed biochemical studies showed it to be attached via a GPI anchor with its expression predominantly localised to the somato-dendritic membrane domain. Thus, a glycolipid anchor could be used to target transgenic proteins selectively to the dendritic plasma membrane of neurons to modify specifically the interactions of neurotransmitter membrane receptor molecules, with the intracellular signalling machinery.

In summary, our results demonstrate that a marker protein, whose only targeting sequence is contained within the glycolipid anchor, can be virally expressed in mammalian cells and correctly targeted to their outer plasma membrane. Thus, in addition to virion, and transcriptional targeting, these data demonstrate that viral vectors could be successfully used to allow the post-transcriptional subcellular targeting of potentially therapeutic transgenes without deleteriously affecting cellular structure and/or function.

Materials and methods

Virus preparation, cell culture and infection

The HSV-1 temperature-sensitive mutant vector *tsK-TT21* was prepared using methodology described previously in detail in Lowenstein *et al.*⁷ Recombinant plasmid pTT1 is also described in Lowenstein *et al.*⁷ and contains the hybrid sequence TIMP/Thy1. pTT1 was cotransfected into baby hamster kidney cells with intact *tsK* DNA by calcium phosphate-mediated precipitation and incubated at the permissive temperature for *tsK* replication of 31°C. Virus progeny containing TIMP/Thy1 inserted into the TK locus were enriched by growth in 100 mg/ml bromodeoxycytidine and purified. The proportion of wild-type revertants within the vector *tsK-TT21* stocks was calculated to below 1×10^5 , below the level of concern for the short-term experiments described in this study.

Ten-day-old primary cultures of neocortical or hippocampal neurons were prepared as described in Lowenstein *et al.*⁷ MDCK cells were grown to confluency using a two chamber culture system in which 10-mm tissue culture inserts (Nunc cat. No. 152752, Nalge Nunc International, Rochester, NY, USA) were placed into a 24-well plate. Once fully polarised the cultures were infected with *tsK-TT21* at a multiplicity of infection (MOI) of 10. Fourteen hours after infection the cells were washed with PBS and then fixed for 30 min in 4% paraformaldehyde/0.12 M sucrose in PBS pH 7.4. After washing the cultures three times with PBS, cells were permeabilised with 0.3% Triton X-100 (BDH Chemicals, Poole, UK) in PBS for 5 min, rinsed and incubated in 10% blocking serum (10% normal horse serum/1% BSA in PBS) for 45 min. After washing with 1% blocking serum (1% normal horse serum/1% BSA in PBS) the cells were immunostained as described elsewhere.⁷

Evaluation of the effect of viral infection on epithelial cell polarity

The protocol was adapted from Caplan *et al.*¹⁸ MDCK cells were grown to confluency in 10-mm tissue culture inserts (Nunc cat. No. 152752) placed into 24-well plates, then cultured for a further 6 days to allow full polarisation. The cells were inoculated at $t = 0$, at a MOI of 10 with *tsK-TT21*. Media alone was added to most wells. The membrane was scratched in control wells to measure ³H inulin transport across a leaky epithelial sheet. To assess the polarity of the MDCK monolayer ³H inulin (*M*₁ 5000, New England Nuclear, Boston, MA, USA, 20 mCi/g) was also added at $t = 0$ to the apical chamber. The cells were incubated at either 37°C (nonpermissive temperature for *tsK-TT21*; no viral replication) or 31°C (permissive temperature for *tsK-TT21*; viral replication) for 48 h. Ten microlitre aliquots of both the basolateral and apical medium were sampled and processed for scintillation counting at 30 min intervals up to 6 h, and then at 12, 24, 30, 36 and 48 h after infection. Integrity of the MDCK monolayer was assessed by measuring the rate at which the radiolabelled macromolecule ³H inulin could pass from the apical compartment to the basolateral compartment.

Antibodies for Western blotting

Mouse monoclonal anti-TIMP was kindly provided by Ian Clarke and Tim Cawston (Rheumatology Unit, Addenbrokes Hospital, Cambridge, UK). Rabbit polyclonal anti-CRD antibody was obtained from Oxford Glycosystems (cat. No. GPI-01, Oxford, UK). Secondary rabbit polyclonal anti-mouse and goat polyclonal anti-rabbit biotin-conjugated secondary antibodies were then used followed by the Vectastain Elite ABC kit (Vector Laboratories cat. No. PK-6100, Peterborough, UK) to label biotinylated proteins and the TIMP and CRD epitopes, respectively.

Antibodies for fluorescence immunocytochemistry

Monoclonal mouse antibodies against microtubule-associated protein (MAP 2), (Sigma cat. No. M-4403, St Louis, MO, USA) and polyclonal rabbit anti-tau antibodies were kindly provided by Professor Brian Anderton and Dr Diane Hanger (Institute of Neurology, London, UK). Mouse monoclonal anti-TIMP was kindly provided by Ian Clarke and Tim Cawston. Secondary antibodies used were rabbit anti-mouse fluorescein or rhodamine-conjugated (DAKO cat. No. F232, F270, High Wycombe, UK) and swine anti-rabbit, rhodamine-conjugated antibody (DAKO cat. No. R156), used in the appropriate combinations for either single or double labelling immunofluorescence.

Biotinylation, PI-PLC cleavage and Western blot analysis

Fourteen hours after infection with *tsK-TT21* (MOI 10) the cells were incubated (30 min, 4°C) in phosphate-buffered saline (PBS) containing 1 mg/ml of sulfo-N-hydroxysuccinimido-biotin (EZ-Link sulfo-NHS-biotin; Pierce cat. No. 21217, Rockford, IL, USA) to biotinylate proteins exposed on the cell surface. The cells were washed thoroughly with PBS/0.1% BSA to quench the excess of sulfo-N-hydroxysuccinimido-biotin. The cells were then pelleted and resuspended in PBS containing 0.8 µg/ml leupeptin and 40 µg/ml pepstatin A to inhibit

peptidases. The sample was then divided into two parts. One sample was stored at 4°C while GPI-linked proteins were detergent extracted from the second sample with precondensed Triton X-114 (45 min, 0°C followed by 30 min, 31°C). The upper aqueous phase was discarded and the Triton phase retained for Western blot analysis. Following this treatment both samples were solubilised in Laemmli sample buffer and run on a 10% PAGE gel under reduced conditions. Western blots were performed on both samples and biotin and human TIMP immunoreactivity assessed.

Alternatively, 14 h after infection with *tsK-TT21* (MOI 10) the cells were treated with 0.34 units/ml of protease-free phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (Oxford Glycosystems cat. No. GPI-02, Oxford, UK) for 60 min at 37°C. Following this treatment, the cells were pelleted and both the cell extract and supernatant were solubilised in Laemmli sample buffer and run on a 10% PAGE gel under reduced conditions. Western blots were performed to assess the human TIMP and cross-reacting determinant (CRD) immunoreactivity.

Selective biotinylation of basolateral membrane proteins

MDCK cells were grown to confluency on 10-mm tissue culture inserts (Nunc cat. No. 152752, Nalge Nunc International) placed into 24-well plates, then cultured for a further 6 days to allow full polarisation. Twelve hours after infection with *tsK-TT21* (MOI 10) the cells were incubated with either of the antibiotics actinomycin D (10 µg/ml) or cycloheximide (50 µg/ml) for 3 h, at 37°C. The cells were then washed thoroughly with PBS/0.1 M CaCl₂/1 mM MgCl₂ (PBS-CM). Basolateral surface proteins were biotinylated by incubation with PBS-CM containing EZ-Link sulfo-NHS-biotin (Pierce cat. No. 21217) at 1 mg/ml added to the basolateral chamber compartment for 40 min, 4°C. Following this treatment the cells were washed with PBS and then fixed for 30 min in 4% paraformaldehyde/0.12 M sucrose in PBS, pH 7.4. After washing the cultures three times with PBS, cells were permeabilised with 0.3% Triton X-100 (BDH) in PBS for 5 min, rinsed and incubated in 10% blocking serum (10% normal horse serum/1% BSA in PBS) for 45 min. After washing with 1% blocking serum (1% normal horse serum/1% BSA in PBS) the cells were immunostained for TIMP using mouse monoclonal anti-TIMP antibody (1:500 in 1% blocking serum). Rabbit anti-mouse rhodamine-conjugated antibody, diluted 1:100 (DAKO cat. No. R270) and avidin-fluorescein, diluted 1:100 were used to label the TIMP monoclonal antibody and biotinylated proteins, respectively.

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