Cell, Vol. 88, 223–233, January 24, 1997, Copyright ©1997 by Cell Press

Intercellular Trafficking and Protein Delivery by a Herpesvirus Structural Protein

Gillian Elliott and Peter O'Hare
Marie Curie Research Institute
The Chart
Oxted, Surrey RH8 0TL
United Kingdom

Summary

We show that the HSV-1 structural protein VP22 has the remarkable property of intercellular transport, which is so efficient that following expression in a subpopulation the protein spreads to every cell in a monolayer, where it concentrates in the nucleus and binds chromatin. VP22 movement was observed both after delivery of DNA by transfection or microinjection and during virus infection. Moreover, we demonstrate that VP22 trafficking occurs via a nonclassical Golgi-independent mechanism. Sensitivity to cytochalasin D treatment suggests that VP22 utilizes a novel trafficking pathway that involves the actin cytoskeleton. In addition, we demonstrate intercellular transport of a VP22 fusion protein after endogenous synthesis or exogenous application, indicating that VP22 may have potential in the field of protein delivery.

Introduction

The trafficking of proteins within the eukaryotic cell is prerequisite for the ordered delivery of individual cellular components to their appropriate compartments. The highly regulated intracellular pathways that utilize the budding and fusion of membrane-bound vesicles for such activities as transport within the cell, secretion out of the cell, and uptake into the cell via endocytosis are now very broadly understood (Rothman, 1994; Rothman and Wieland, 1996). Nascent proteins destined for trafficking are targeted cotranslationally to the lumen of the endoplasmic reticulum (ER) by an N-terminal hydrophobic signal sequence. Vesicles containing these proteins then bud from the ER and are conveyed to the Golgi apparatus where they are sorted to their ultimate destination of either internal organelles or the plasma membrane. On the inward path, endocytosis involves the internalization of extracellular molecules through the invagination and subsequent pinching of vesicles from the cell surface, which are then conveyed to the endosomal compartment.

Together with many other aspects of cell biology, the field of protein trafficking has been significantly enhanced by studies carried out on eukaryotic viruses. We have been interested in protein trafficking in herpes simplex virus type 1 (HSV-1) infected cells and in the features involved in targeting and selective incorporation into different virion compartments during virus assembly. In particular, we have been investigating the properties and subcellular localization of a group of structural proteins that make up the poorly understood region of the virion located between the capsid and the envelope, which is termed the tegument (Dargan, 1986). In this paper we show that one of these proteins, the structural protein VP22 (Elliott and Meredith, 1992), exhibits a combination of features that distinguishes it as a protein with novel trafficking properties. Like a small number of unusual proteins, such as interleukin 1β (Rubartelli et al., 1990), the HIV-1 Tat protein (Ensoli et al., 1993), and the fibroblast growth factors (FGFs) (Jackson et al., 1992), VP22 is exported from cells in which it is synthesized, in spite of lacking a signal sequence, by a Golgi-independent mechanism termed nonclassical secretion (Kuchler, 1993). VP22 is unique, however, in its ability to reenter surrounding cells with such a high level of efficiency that, after endogenous synthesis in a small subpopulation of cells, the protein formed gradients in surrounding recipient cells and could spread to virtually every cell in a transfected monolayer. VP22 movement was also observed after delivery of DNA by microinjection and after infection with a disabled nonreplicating virus. Moreover, in recipient cells VP22 was directed to the nucleus, despite lacking a nuclear localization sequence, where it bound to chromatin and segregated to daughter cells. We show that uptake of VP22 does not involve the standard route of endocytosis, but appears to involve a novel pathway that is cytochalasin D sensitive and therefore would seem to require an intact actin cytoskeleton. Finally, we show that by modification of VP22, both a peptide and a 27 kDa protein could be delivered to all cells in a monolayer after endogenous expression, the first time such delivery has been documented. Taken together, these features suggest that VP22 will be both an extremely useful tool for analyzing nonclassical transport within the eukaryotic cell, and a potential vector for protein delivery.

Results

Heterogeneity in VP22 Cellular Localization

As part of our program to characterize the structural tegument proteins of HSV-1, we initiated a detailed analysis of one of the major proteins of this compartment, the 38 kDa protein VP22 (Figure 1A). To examine its pattern of independent subcellular localization, COS-1 cells were transfected with a VP22 expression vector and analyzed by indirect immunofluorescence using the monoclonal antibody P43 (Elliott and Meredith, 1992). Consistent with our previous results (Elliott et al., 1995), P43 does not cross-react with untransfected cells (Figure 1B, mock), while the predominant pattern observed in transfected cells was that of nuclear staining, frequently with enrichment around the nuclear rim (Figure 1B, WT VP22, left-hand panel). In a number of cells, however, VP22 was present in a distinctive cytoplasmic filamentous pattern (Figure 1B, WT VP22, right-hand panel). We further noted that these two patterns were always detected in adjacent cells, with one cell containing cytoplasmic VP22 surrounded by several cells containing nuclear VP22 (Figure 1C, WT VP22). Comparison of the cellular localization of a VP22 mutant that

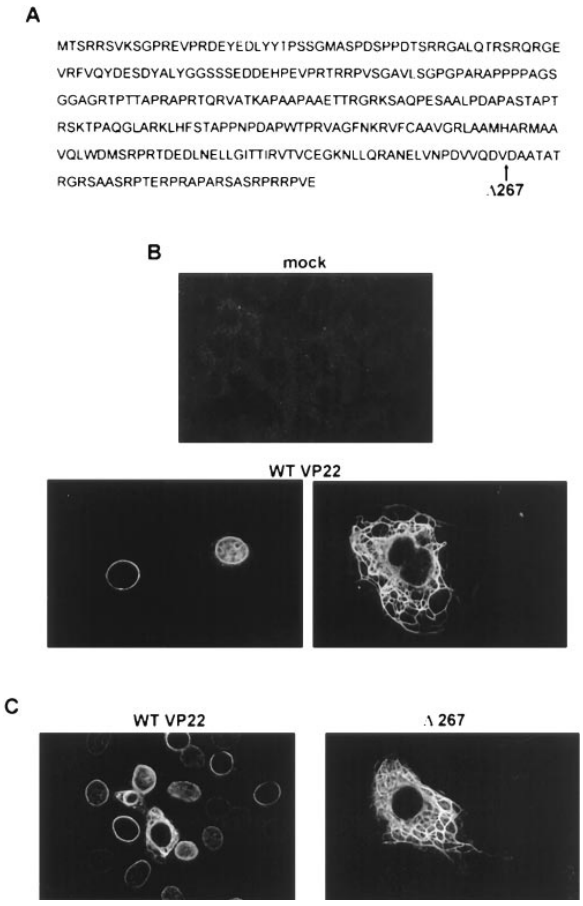

Figure 1. Cellular Localization of HSV-1 VP22 during Transient Expression

(A) Coding sequence of VP22, the product of the HSV-1 UL49 gene, showing the truncation point for the Δ267 mutant.

(B) Cell-staining patterns observed with antibody P43 for untransfected COS-1 cells (mock) and pGE109 transfected COS-1 cells (WT VP22).

(C) Comparison of VP22 localization patterns observed with P43 in cells transfected with pGE109 (WT VP22), and the C-terminal deletion mutant (Δ267).

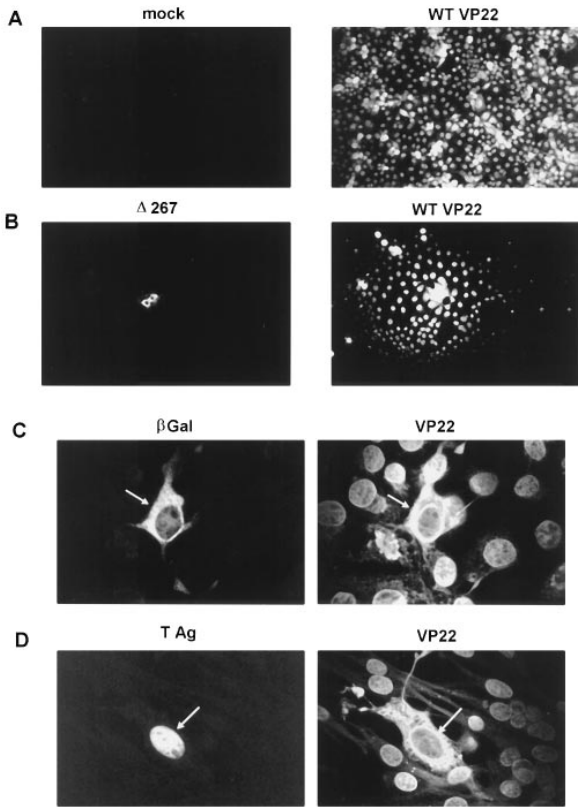

Figure 2. VP22 Spreads between Cells during Transient Expression

(A) Cell-staining pattern observed at low magnification with antibody AGV30 for untransfected COS-1 cells (mock) and pGE109 transfected cells (WT VP22).

(B) Cell-staining patterns observed with antibody AGV30 using 10-fold less DNA for transfection than in (A) for both pGE109 (WT VP22) and the C-terminal deletion mutant (Δ267). In (A) and (B) images were collected using a 10× objective.

(C) COS-1 cells were microinjected with a mixture of plasmids pGE109 and pBAG, incubated for 24 hr, and double immunofluorescence carried out with the anti-β-galactosidase antibody (β-Gal), and AGV30 (VP22). The same field is shown in both panels and the injected cell is arrowed.

(D) COS-1 cells transfected with pGE109 were mixed with untransfected BHK-21 cells at a ratio of 1:20, and 20 hr later double immunofluorescence carried out with the anti–T antigen antibody pAB419 (T Ag) and AGV30 (VP22). The same field is shown in both panels with the COS-1 cell expressing VP22 arrowed.

lacks the C-terminal 34 residues (Δ267, Figure 1A) demonstrated that this striking characteristic of VP22 localization in cell clusters was specific to the full-length protein. While Δ267 was synthesized in transfected cells at similar levels to WT VP22, as judged by Western blot (e.g. see Figure 4B), the mutant VP22 protein was only found in the cytoplasmic filamentous pattern, and was not detected in the nuclei of surrounding cells (Figure 1C, Δ267). Furthermore, the numbers of cells containing VP22 within the cytoplasm were approximately the same for the mutant and WT proteins. This specific relationship of a central cell with cytoplasmic VP22 immediately surrounded in a nonrandom manner by cells with nuclear VP22, together with the phenotype of a mutant with only the cytoplasmic pattern suggested that VP22 exhibited an unusual property whereby the protein localized to the cytoplasm of the cell in which it was initially expressed and was transported to adjacent cells where it accumulated in the nuclei. Moreover, the observation that the C-terminal deletion mutant localized only in the cell of expression indicated that a determinant at the extreme C-terminus of VP22 may be essential for its transport function.

VP22 Can Spread into the Nuclei of All Cells in a Tissue Culture Monolayer

To investigate further the movement of VP22 between cells, we utilized an independent polyclonal antibody to VP22 (AGV30) to define the proportion of cells containing VP22 following transfection. As expected, this antibody did not cross-react with untransfected COS-1 cells (Figure 2A, mock). Strikingly, however, immunofluorescence of transfected cells examined at low magnification clearly showed that VP22 was present within every cell

of the monolayer (Figure 2A, WT VP22), a property not observed with any other test protein we have compared. Within these monolayers, an approximate 10% subpopulation contained VP22 in its bright cytoplasmic staining, while the remainder contained lower intensity nuclear VP22. To analyze the efficiency of VP22 spreading, we used 10-fold less plasmid DNA such that a field containing only a few expressing cells could be observed at low magnification. The Δ267 mutant was examined in parallel, and typical results are shown in Figure 2B. The Δ267 mutant VP22 protein was only detectable in the two transfected cells within the field (Figure 2B, Δ267). In contrast, transfection of WT VP22 under the same conditions resulted in a gradient of VP22 emanating from a single transfected cell in the center of the field into a halo of surrounding cells, which had a diameter of up to 16 cells (Figure 2B, WT VP22). This powerful image both demonstrates that in the course of transfection one VP22-expressing cell synthesizes protein that is destined to reach at least 200 other cell nuclei, and provides the explanation for the ability of VP22 to reach every cell in a transfected monolayer from a subset of expressing cells (Figure 2A).

To prove that VP22 possesses the ability to move between cells, we used microinjection as an alternative technique of introducing plasmid DNA into cells. The plasmid encoding VP22 was injected into the nuclei of individual COS-1 cells, together with an expression plasmid for β-galactosidase to confirm and mark the single injected cells. Typical results are shown in Figure 2C. The injected cells were easily distinguishable by their reaction with an anti-β-gal antibody (arrowed in Figure 2C, β-gal), which did not react with any of the surrounding cells. The injected cell also contained VP22 within its cytoplasm (arrowed in Figure 2C, VP22), but in striking contrast to β-galactosidase, VP22 was present not only in the injected cell but also in the nuclei of all the surrounding cells (Figure 2C, VP22). These experiments confirm the results obtained by DNA transfection and prove conclusively that VP22 has the ability to spread from the original cell of expression into neighboring cells in a manner so efficient that it is detectable by immunofluorescence.

The experiments described so far were all carried out in COS-1 cells, but transfection of other cell-types, such as BHK-21, Vero, or HeLa, also yielded the same results (data not shown), demonstrating that VP22 movement is not specific to COS-1 cells. Moreover, we were able to demonstrate intertypic cell spreading of VP22 by conducting the following experiment: COS-1 cells transfected with the VP22 expression vector were trypsinized 24 hr after transfection and mixed at a ratio of 1:20 with trypsinized nontransfected BHK-21 cells. The mixed cell population was then plated and incubated for a further 24 hr. The COS-1 cells in this mixed monolayer were easily identified by staining for large T antigen (arrowed in Figure 2D, T Ag). The same field stained for VP22 showed that VP22 synthesized in the original transfected COS-1 cell (arrowed in Figure 2D, VP22) moved into the nuclei of surrounding BHK-21 cells (Figure 2D, VP22). No T antigen was observed in the BHK cells. Thus, an additional feature of VP22 is that the protein can spread between different cell-types.

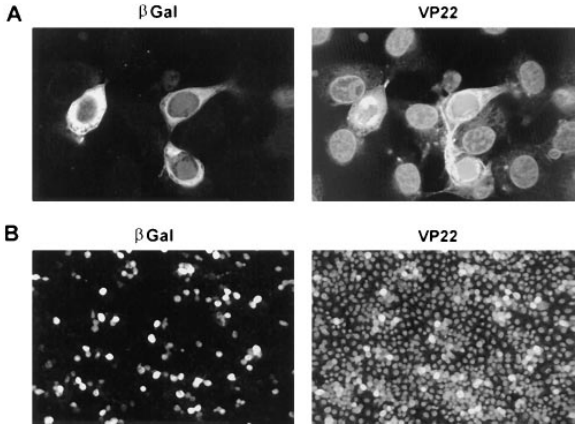

Figure 3. VP22 Is Transported during Virus Infection

(A) and (B) COS-1 cells were infected with the gH⁻ mutant of HSV-1 at a multiplicity of 0.1, and double immunofluorescence carried out 30 hr later using anti-β-galactosidase antibody (β-Gal), and AGV30 (VP22). Images taken at high (A) and low (B) magnification are shown, and the same field stained for both proteins is shown in each case.

VP22 Is Transported between Cells during Virus Infection

The role of VP22 during HSV-1 infection is as yet unclear, but if the protein moves between cells during the course of a virus infection, it is likely that this property will be relevant to some aspect of the virus replication cycle. In determining if VP22 exhibits intercellular movement during infection, it was necessary to ensure that, within any candidate VP22 recipient cell, VP22 was not being synthesized de novo but had originated from a separate virus-infected cell. We therefore investigated the spread of VP22 in cells infected with a disabled HSV-1 mutant in which the gene for the glycoprotein gH, known to be essential for virus entry into the cell (Gompels and Minson, 1986; Fuller et al., 1989), had been replaced by the gene encoding β-galactosidase (Forrester et al., 1992). When the mutant virus is grown on a gH-expressing cell line, infectivity is retained due to complementation by the endogenous gH protein that is incorporated into virions. However, when this complemented virus is used to infect a noncomplementing cell line, the virus can only initiate the original round of infection, and secreted progeny virus particles cannot enter cells to initiate a second round of infection. Thus, in normal cells infected with this mutant, viral protein synthesis and assembly are limited to the primary infected cell, and any movement of VP22 observed in these cells must be due to a mechanism other than uptake of infectious virus. A monolayer of 5×10^5 COS-1 cells was infected with complemented gH⁻ virus at a mutiplicity such that one in ten cells would be infected. Thirty hours later the cells were fixed and stained for both β-galactosidase (as a means of identifying infected cells) and VP22. The infected cells stained intensely for β-galactosidase (Figure 3A, β-gal) and other viral proteins (data not shown). The VP22 staining in the infected cells was also intense, but unlike β-galactosidase, VP22 was also detectable in the nuclei of all the uninfected cells (Figure 3A, VP22). This phenomenon was clearly demonstrated when the cells were examined at low magnification (Figure 3B),

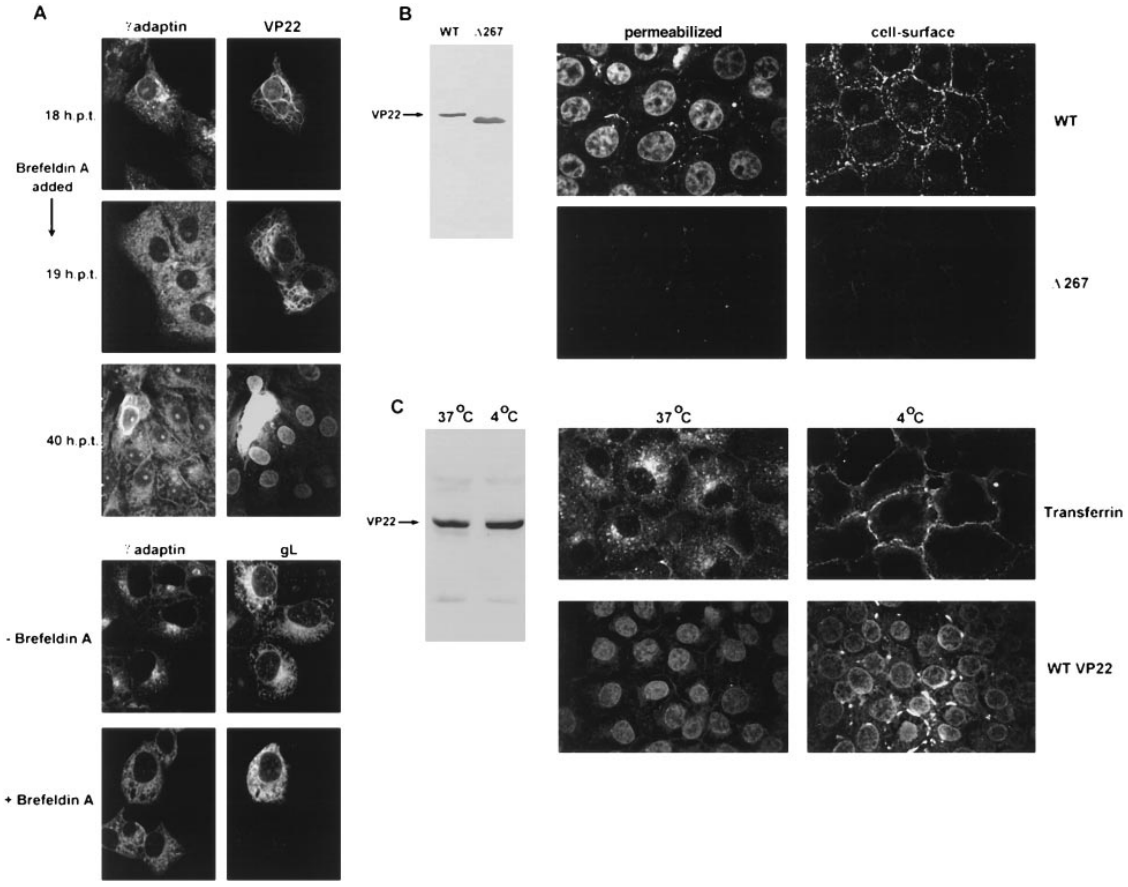

Figure 4. Transport of VP22 Occurs via a Nonclassical Mechanism

A) VP22 movement does not require functional Golgi transport. Vero cells transfected with pGE109 were incubated for 18 hr, Brefeldin A added (2.5 μg/ml), and incubated for a further 22 hr in the presence of the inhibitor. Samples were taken at 18 hr (prior to addition of Brefeldin A), 19 hr (1 hr after addition of Brefeldin A) and 40 hr, and double immunofluorescence carried out with AGV30 (VP22) and the anti-γ-adaptin monoclonal antibody (γ-adaptin). The bottom two panels show COS-1 cells transfected with a gL expression vector, incubated in either the absence or presence of Brefeldin A (2.5 μg/ml) as described for the top three panels, and stained for both γ-adaptin and gL.

(B) Exogenous VP22 is taken up by cells. The left-hand panel shows a Western blot of cell-free extracts prepared from pGE109 (WT) and Δ267 transfected COS-1 cells (Δ267), reacted with antibody AGV 30. Equal amounts of these extracts were added to the medium covering 5×10^5 COS-1 cells on coverslips. The cells were fixed 1 hr later and stained with the antibody AGV30. Examples are shown of both cell-surface staining and permeabilized intracellular staining.

(C) Uptake of VP22 does not involve classical endocytosis. COS-1 cells were incubated for 15 min at 4°C, and either a VP22-containing extract (WT VP22), or Texas red–conjugated transferrin (Transferrin) added to the medium. The cells were maintained at 4°C for 1 hr and fixed for immunofluorescence. Both VP22 and Texas red-transferrin were also added to cells at 37°C in parallel experiments. Samples of COS-1 cells exposed to the WT-VP22 extract at both 4°C and 37°C were also used to prepare nuclear extracts, which were analyzed by Western blotting using antibody AGV30 (left-hand panel).

where it can be seen that, while only ~10% of the cells had been infected, VP22 was present within every cell in the monolayer. This confirms that VP22 has the capacity to spread between cells during infection.

VP22 Movement Does Not Involve the Golgi Apparatus or Classical Endocytosis

Analysis of the VP22 open reading frame (Figure 1A) revealed that it does not possess a hydrophobic N-terminal signal sequence and therefore was unlikely to be transported through the classical ER/Golgi route. To investigate the role of the Golgi in VP22 transport we used the drug Brefeldin A, which is known to disrupt the Golgi apparatus and inhibit classical vesicle-mediated secretion (Lippincott Schwartz et al., 1990). We transfected Vero cells with the VP22 expression vector and added Brefeldin A 18 hr later, at a time when VP22 had not yet left the original cells of expression (Figure 4A, top panel, VP22). As a control for Golgi disruption, the cells were also stained for a Golgi marker, namely the cellular protein γ-adaptin (Robinson and Kries, 1992), a component of nonclathrin-coated vesicles that concentrates in the Golgi (Figure 4A, top panel, γ-adaptin). The action of Brefeldin A was almost immediate, as shown by the diffuse cytoplasmic staining of γ-adaptin 1 hr after addition of the drug (Figure 4A, second panel, γ-adaptin). However, after a further 20 hr exposure to the drug, and with the Golgi still disrupted (Figure 4A, third panel, γ-adaptin), VP22 had moved from the cell of synthesis into the nuclei of surrounding cells (Figure

4A, third panel, VP22). As an additional control to the γ-adaptin localization, we transfected cells with a vector expressing the HSV-1 glycoprotein gL, which contains a signal sequence for Golgi targetting (Hutchinson et al., 1992). Following transfection, cells were treated with Brefeldin A as before and stained for γ-adaptin and gL (Figure 4A, bottom two panels). In untreated cells gL was localized in the Golgi in a manner similar to γ-adaptin. After exposure to Brefeldin A, however, gL was relocalized throughout the cytoplasm of expressing cells, confirming that treatment with the drug blocked the trafficking of a signal sequence–containing protein. These results, together with the Brefeldin A resistance of VP22 transport, indicate that VP22 does not require an intact Golgi for transportation between cells and, consistent with its lack of a signal sequence, is likely to be secreted by a nonclassical mechanism.

Our results indicated that VP22 was either exported from one cell into the medium from where it was taken up by surrounding cells, or that it moved across intercellular contacts. To examine the ability of VP22 to be imported from the medium, a soluble high-salt extract made from VP22-expressing cells was added to the medium of untransfected cells. Under these conditions VP22 was imported and localized to the nucleus rapidly and efficiently (Figure 4B, WT VP22, permeabilized sample), with VP22 detectable in the nuclei of cells within 5 min after addition to the medium. Moreover, the VP22 deletion mutant Δ267, while present in the soluble extract in equivalent amounts to full-length VP22 (Figure 4B, left-hand panel), was never detected intracellularly (Figure 4B, Δ267, permeabilized sample). Staining of nonpermeabilized cells demonstrated WT VP22 on the surface of cells, while the Δ267 protein could not be detected (Figure 4B, cell-surface samples), implicating the C-terminal 34 residues in VP22 attachment to cells.

We next addressed the mechanism of VP22 uptake by cells. The standard route of entry for proteins into cells is receptor-mediated endocytosis, where proteins bind to specific receptors on the cell-surface, and these ligand/receptor complexes are internalized in clathrin-coated membrane vesicles. It was therefore of interest to determine if VP22 was imported by classical endocytosis by testing its sensitivity to incubation at 4°C, a treatment well established to inhibit endocytosis (Pastan and Willingham, 1981). As a control, we tested import of Texas red–conjugated transferrin that is imported via the transferrin receptor and classical receptor-mediated endocytosis. As expected, transferrin was taken up by cells at 37°C into vesicles located within the cytoplasm (Figure 4C, transferrin at 37°C). At 4°C, endocytosis of the transferrin was completely inhibited, and the protein exhibited a complete qualitatively distinct pattern, remaining bound to its receptor on the cell surface (Figure 4C, transferrin at 4°C). In contrast, import of VP22 at 4°C was little altered in comparison to that detected at 37°C (Figure 4C, WT VP22, compare 37°C and 4°C), a result confirmed by Western blotting of nuclear extracts made from cells treated in the same manner (Figure 4C, left-hand panel). These results suggest that internalization of VP22 is energy-independent and occurs via a route other than normal endocytosis.

VP22 Movement between Cells Involves the Actin Cytoskeleton

The fact that a protein enters a cell and trafficks efficiently and rapidly to the nucleus, without using the endocytotic pathway, suggests that it may utilize an alternative connection between the cell surface and the nucleus, rather than simply diffusing through the cell. The cytoskeleton of the cell could provide one such pathway, with a combination of microtubules, intermediate filaments, and actin microfilaments forming a complex network of interconnected filaments running from the cell surface through to the nuclear membrane. Moreover, the filamentous pattern of VP22 localization observed in VP22-expressing cells suggested that it may have the potential to interact with one or more of the components of the cytoskeleton. Thus, we investigated the role of the individual components of the cytoskeleton in the VP22 import assay. First, to determine if the microtubule network was involved in VP22 trafficking, we utilized the microtubule depolymerizing drug nocodazole. In the absence of nocodazole treatment, the microtubule network was intact within the cell (Figure 5A, α-tubulin staining, − noc), and VP22 was imported into the nucleus as described before (Figure 5A, VP22, − noc.). Pretreatment of cells with nocodazole prior to addition of the VP22 extract to the extracellular medium, resulted in the depolymerization of the microtubule network (Figure 5A, α-tubulin staining, + noc). VP22 was still imported efficiently, however, into the nuclei of these cells (Figure 5A, VP22 + noc), indicating that intact microtubules are not required for VP22 import.

We next utilized the drug cytochalasin D to target the microfilament network within the cell. Pretreatment of cells with this microfilament-disrupting drug, prior to addition of the VP22-containing extract, resulted in a very different pattern of VP22 localization within the cell. While a proportion of VP22 was taken up and targeted to the nucleus (Figure 5B, + cyto D permeabilized sample), the level of import was less than that observed in untreated cells (Figure 5B, − cyto D). Furthermore, in cytochalasin D–treated cells VP22 localized around the cell surface in large aggregates (Figure 5B, + cyto D, permeabilized and cell-surface staining, cf. Figure 4C), implying that cytochalasin D treatment had dramatically altered the cell-surface binding sites of VP22.

To investigate further this proposal, we examined the effect of cytochalasin D treatment on VP22 transport following endogenous synthesis during transfection. COS-1 cells were treated with cytochalasin D 8 hr after transfection, at a time when VP22 had not yet moved from the original cell of expression. Twelve hours later the cells were fixed and assessed for VP22 movement between cells. In untreated cells, VP22 had already moved into all the surrounding nuclei from the expressing cell (Figure 5C, − cyto D). In the cells treated with cytochalasin D, however, there was no evidence for VP22 movement from the original expressing cells (Figure 5C, + cyto D). A series of control experiments demonstrated that treatment with cytochalasin D had no effect on the level of VP22 within the cells or its integrity (Figure 5D), confirming that the lack of VP22 movement after treatment with the drug was not due to a reduced level of synthesis or degradation of the protein. Moreover, cytochalasin D–treated cells were able to import

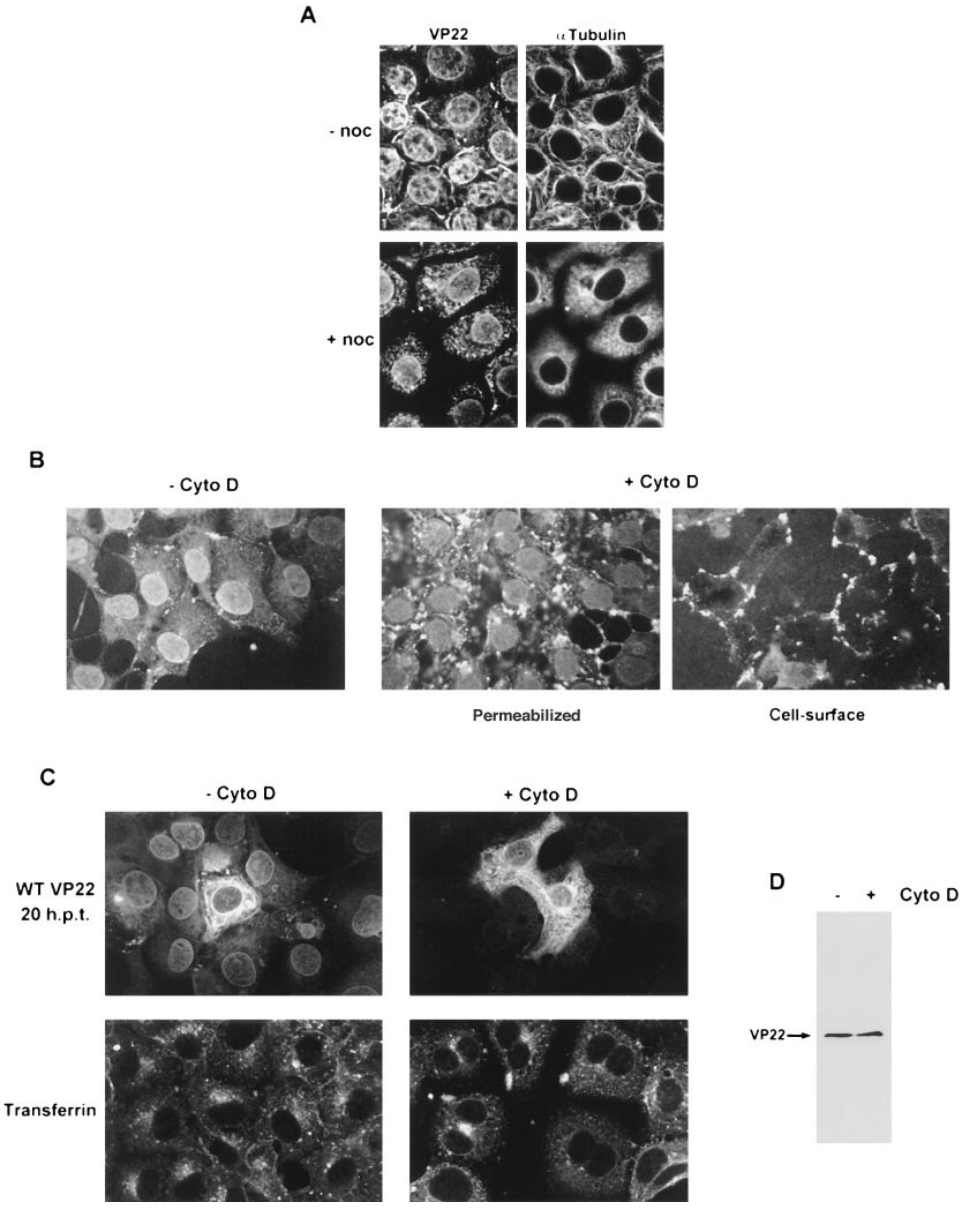

Figure 5. A Role for the Cell Cytoskeleton in VP22 Uptake

(A) Prior to addition of a VP22-containing extract, COS-1 cells were incubated for 30 min in the absence (− noc) or presence (+ noc) of 10 μg/ml nocodazole. Following addition of the extract, the cells were maintained in these conditions for 1 hr, and then fixed and stained with both AGV30 (VP22), and the anti-α-tubulin antibody (α-tubulin).

(B) COS-1 cells were incubated for 15 min either in the absence (− cyto D) or presence (+ cyto D) of the drug cytochalasin D (1 μM). A VP22-containing extract was then added, and incubated in the same conditions for a further hour, whereupon the cells were fixed and stained with AGV30. Examples are shown for both internal staining (Permeabilized) and cell-surface staining (Cell-surface) in the cytochalasin D-treated cells.

(C) COS-1 cells transfected with pGE109 were incubated for 8 hr, and then cytochalasin D (1 μM) added to the medium. The cells were incubated for a further 18 hr in the presence of the drug before being fixed and stained with AGV30 (+ cyto D). Cells treated in an identical manner, but in the absence of cytochalasin D treatment, are also shown (− cyto D). COS-1 cells incubated in cytochalasin D for 18 hr were also exposed to Texas red-transferrin (Transferrin) for 1 hr, and examined for uptake.

(D) COS-1 cells treated as described in (C) were solubilized and analyzed by Western blotting with antibody AGV30.

Texas red–conjugated transferrin as efficiently as untreated cells (Figure 5C, Transferrin), indicating that cytochalasin D had not caused an obvious pleiotropic effect on the cells. These results demonstrate that the actin microfilament network is involved in VP22 transport.

Delivery of Heterologous Polypeptides by VP22

These remarkable properties of potent transport and nuclear targeting suggested that VP22 may represent a realistic polypeptide delivery system. To investigate the potential of VP22 to transport additional residues between cells, a vector containing the VP22 open reading

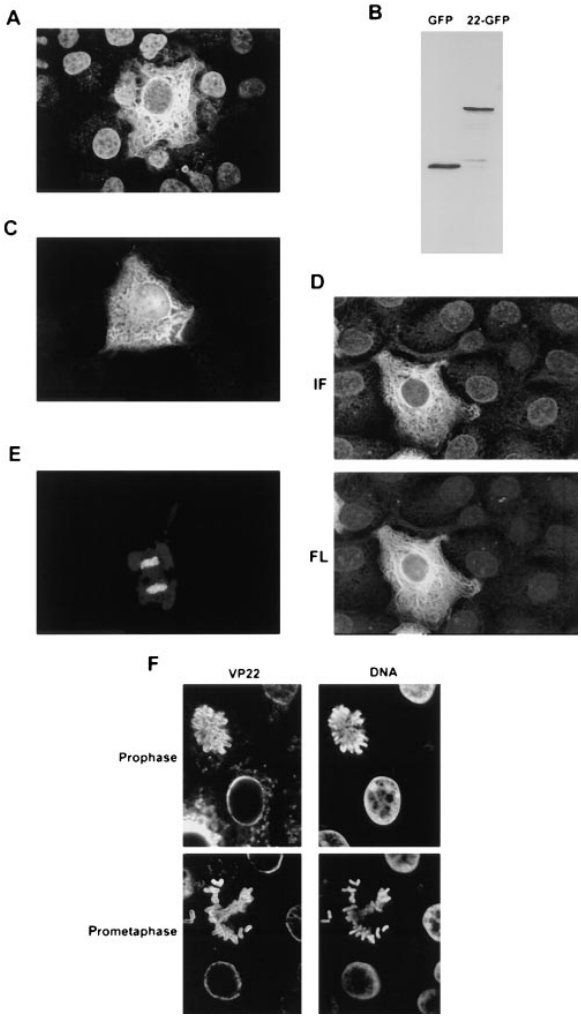

Figure 6. VP22 as a Delivery Vehicle for Heterologous Polypeptides

(A) COS-1 cells were transfected with the plasmid UL49ep, which expresses a VP22-peptide fusion protein. Forty hours after transfection the cells were fixed and stained with antibody AGV30.

(B) COS-1 cells were transfected with either plasmid pEGFP, which expresses the GFP protein, or pGE150, which expresses a VP22-GFP fusion. The cells were harvested 40 hr after transfection and analyzed by Western blotting using the anti-rGFP antibody.

(C) COS-1 cells transfected with pEGFP were fixed after 40 hr, and immunofluorescence carried out with anti-rGFP.

(D) COS-1 cells transfected with pGE150 were fixed after 40 hr, and analyzed by immunofluorescence with anti-rGFP (IF) and by direct GFP fluorescence (FL). Both panels show the same field.

(E) Live cell fluorescence of COS-1 cells expressing 22-GFP. This example shows a cell going through mitosis.

(F) COS-1 cells transfected with pGE109 were double labeled with antibody P43 (VP22) and propidium iodide (DNA). Cells at the mitotic stages of prophase and prometaphase are shown, with the same field shown in each panel.

frame fused at its C-terminus to a 12-residue peptide, i.e., plasmid UL49ep (Leslie et al., 1996), was transfected into COS-1 cells, and analyzed by immunofluorescence. Staining with the antibody AGV30, which is specific to the N-terminus of VP22 (Figure 6A), or an antibody directed against the C-terminus peptide (data not shown), revealed that this fusion protein was indeed transported intact, with an efficiency similar to that of WT, such that the peptide tag could be detected within every cell in the monolayer. It is also noteworthy that the fusion protein had an identical pattern of localization to WT VP22, exhibiting efficient nuclear targeting.

We also tested the ability of VP22 to import this 12-residue peptide by exogenous delivery. An import assay was carried out as described above, using a soluble extract from cells expressing the VP22-peptide fusion protein. The efficiency of import of the fusion protein was identical to that of WT VP22 (data not shown), suggesting that the additional peptide did not interfere with VP22 uptake. Taken together, these results demonstrate that VP22 can efficiently deliver heterologous peptides into cells either after endogenous synthesis and transport, or by application to the medium and uptake.

Based on these results, we investigated further the potential for delivering much larger intact reading frames. VP22 was fused at its C-terminus to the coding sequence of the 27 kDa jellyfish green fluorescent protein (GFP) (Chalfie et al., 1994) to produce a fusion protein of around 65 kDa. When this fusion protein was expressed in COS-1 cells, it was synthesized intact, as demonstrated by Western blotting with the antibody anti-rGFP (Figure 6B). The subcellular localizations of parental GFP and the 22–GFP fusion protein were then determined using the anti-rGFP antibody. GFP expressed in COS-1 cells exhibited a diffuse cytoplasmic and nuclear localization (Figure 6C), and was only detectable in the original cell of expression. However, fusion of GFP to the VP22 reading frame dramatically altered the localization of GFP in the expressing cell from its diffuse pattern to the filamentous cytoplasmic pattern observed previously for VP22 (Figure 6D, top panel). Moreover, the VP22-GFP fusion protein had clearly moved into the surrounding untransfected cells where, like WT VP22, the fusion protein was located in the nucleus of the cell. This transported protein was detected by antibodies to both the VP22 and the GFP elements of the fusion protein, confirming that the protein was intact. Furthermore, direct GFP fluorescence of the VP22 fusion was also detectable in the nuclei of the same field of cells (Figure 6D, lower panel). Thus, VP22 has the capacity to deliver a 27 kDa protein into the nuclei of adjacent cells following endogenous synthesis.

In spite of the clearly efficient targeting of VP22 to the nuclei of recipient cells, there is no recognizable consensus nuclear localization signal within the VP22 sequence, although it does contain many basic residues (Figure 1A). While examining the nuclear localization of VP22 under high magnification, we noted that in a proportion of cells it was present in a pattern similar to that of mitotic chromatin (eg. Figure 2C, VP22, bottom left-hand corner of microinjected cell). We therefore performed dual staining of cells for both VP22 and DNA. The results revealed that cells at all stages of mitosis could be detected with VP22 localized around the condensed chromosomes (Figure 6F, examples shown of prophase and prometaphase). Moreover, binding to chromatin at all stages of mitosis was also observed for the VP22–peptide fusion (data not shown) and the VP22–GFP fusion (e.g. Figure 6E). Thus, VP22 is capable of binding either directly or indirectly to chromatin, an

interaction that may help explain its efficient localization to the nucleus. Chromatin binding and segregation to daughter cells are not altered by the addition of a large polypeptide to the C-terminus of VP22.

Discussion

In this paper we demonstrate that the HSV-1 structural protein VP22 exhibits a combination of properties that distinguish it as an extremely unusual protein. VP22 exhibits the remarkable ability to spread between cells so efficiently that after synthesis in only a small proportion of cells the protein accumulates within every cell in a monolayer. VP22 moves from the cell in which it is synthesized, and where it localizes largely in the cytoplasm, to adjacent cells where it is imported and targeted to the nucleus. This movement establishes gradients of VP22 extending outward from the initially expressing cell. Moreover, VP22 movement occurs not only when the protein is expressed individually, but also during virus infection, implying that the property is biologically relevant. Neither VP22 export nor VP22 import involve the classical routes of vesicular trafficking, but appear to involve a novel microfilament-dependent route. Significantly, we have shown that peptides and proteins of up to 27 kDa can be delivered to cells as VP22 fusion proteins by either endogenous synthesis or after application to the culture medium.

Mechanisms of Protein Trafficking

Most proteins that are exported from the cell possess signal sequences and are secreted via the Golgi apparatus. By contrast, a small group of proteins, which, like VP22, lack signal sequences, has been reported to be transported by unknown Golgi-independent mechanisms, including interleukin 1β, the FGFs, and HIV-1 Tat (Kuchler, 1993; Rubartelli and Sitia, 1995). However, thorough analyses of the literature concerning such nonclassical pathways reveal that there are significant differences between these proteins and VP22. For example, interleukin 1β is only secreted by specialized cells and then only in response to stimulation. Little or no secretion was observed in, e.g., COS cells without the addition of a conventional leader sequence (Rubartelli et al., 1990; Krasney and Young, 1992). Likewise, it has been shown that the FGFs remain intracellular when expressed in tissue culture cells (Cao and Pettersson, 1990), while their fate following cellular uptake remains controversial (Baldin et al., 1990; Cao et al., 1993). Although the 87-residue Tat protein has been reported to have unusual transport properties (Helland et al., 1991; Ensoli et al., 1993), in our hands spread of the protein between cells was generally restricted to the nearest neighbors of the initial Tat-expressing cell. Moreover, after exogenous supply, Tat was localized specifically to the cytoplasm, in spite of possessing a well-defined nuclear localization signal (data not shown). Transactivation by Tat under these conditions has been documented as being extremely weak (Ensoli et al., 1993) and may therefore be due to either only a very few molecules of the protein reaching the nucleus, or activation via an indirect cytosolic pathway such as the recently documented Tat activation of NF-κB (DeMarchi et al., 1996). In addition, one other example, the antennapedia homeodomain, has been reported to be taken up by cells after exogenous supply (Joliot et al., 1991; Derossi et al., 1994). Unlike VP22, however, only a subregion of the intact antennapedia protein is taken up by cells, and there is no evidence that the protein is secreted after endogenous synthesis, suggesting that neither export nor import are features of the intact protein.

The cellular mechanisms involved in release and uptake of such proteins are not yet understood. Unlike VP22, interleukin 1β is found within membrane-bound vesicles within the cytoplasm (Rubartelli et al., 1990). It has been suggested that these vesicles represent recycling endosomes and that release of interleukin 1β from human monocytes may involve its sequestration by the endosomal pathway; for although resistant to Golgi disruption, treatments known to interfere with the endosomal compartment inhibit its release (Rubartelli et al., 1990). With regard to uptake, exogenous FGFs and Tat have been shown to enter cells via receptor-mediated endocytosis (Mann and Frankel, 1991; Cao et al., 1993), such that they become internalized and enter the incoming endosomal pathway. In contrast, we have shown that VP22 does not enter cells through endocytosis, but utilizes a novel pathway of entry that appears to involve the actin cytoskeleton. Moreover, we have identified a determinant at the extreme C-terminus of VP22 that is absolutely essential for cell binding and entry, indicating that this region is involved in the interaction between VP22 and its cellular receptor. Because of the requirement for actin in VP22 uptake, we postulate that its receptor may be one of the cell-surface proteins that make contact either directly or indirectly with actin microfilaments. A precedent for such an interaction exists in the recently demonstrated association between the internalin protein of L. monocytogenes, a bacterium that utilizes actin to move through the cell, and its receptor, i.e., the cell–cell adhesion molecule E-cadherin (Mengaud et al., 1996). Our results on the sensitivity of VP22 transport to cytochalasin D treatment cannot distinguish between the alternative situations of both export and import being inhibited, or only import being inhibited. Thus, formally the possibility exists that the export pathway involves an actin-independent mechanism.

VP22 and Virus Replication

Our evidence that movement of VP22 occurs during infection raises the possibility that this function is part of the normal viral activity of the protein. VP22 is known to be modified by phosphorylation and nucleotidylation in the infected cell (Elliott and Meredith, 1992; Blaho et al., 1994), although neither the relevance of these modifications nor the function of VP22 within the infectious cycle are as yet understood. While current results from our attempts to isolate a viral mutant in VP22 imply that it may be essential, demonstrating that the transport role per se is essential will require separation of transport determinants from other possible functional determinants. Although we do not yet understand the precise role of VP22 transport, it is tempting to speculate that during the course of infection VP22, which is first detectable at around 4 hr after infection, would move between

cells at times prior to the production of infectious virions. Once in the nuclei of the neighboring cells, VP22 could act by some unknown mechanism to facilitate the ensuing infection. This mechanism could take the form of alteration of the nuclear architecture, interference with the cell-cycle, or activation/downregulation of cellular genes. These novel results demonstrating a protein gradient after HSV infection may contribute to an understanding of mechanisms involved in virus pathogenesis, and may expand the cellular mechanisms whereby signaling gradients, e.g. in development processes, are established.

VP22 in Gene Therapy

Many of the properties we have described for VP22 make it an attractive candidate for a novel cellular delivery system. Notably, the protein can enter cells either when synthesized endogenously or when supplied exogenously, making it appropriate for a variety of therapeutic applications. While several proteins have been proposed as vehicles for exogenous delivery, and, for example, Tat has been successfully used to import attached proteins from the medium (Fawell et al., 1994), VP22 appears to be novel in possessing unusually potent delivery properties following endogenous synthesis. Moreover, fusion of a 27 kDa protein to the C-terminus of VP22 allowed that peptide to be delivered to cells either by endogenous or exogenous supply, confirming that VP22 has the capacity to act as a vehicle for delivery. In addition, endogenous synthesis following microinjection, a process easily envisaged as a mechanism of delivery in vivo, resulted in the spread of VP22 as effectively as that seen during transfection, where VP22 synthesized in one cell can reach up to 200 other cells. Import of the protein is rapid and efficient, resulting in the delivery of VP22 to the nucleus. Neither the nuclear targeting nor chromatin binding is altered by the addition of a peptide or even a 27 kDa protein, suggesting that VP22 has the potential to deliver factors specifically to the nuclear compartment. In addition, VP22 movement and import do not appear to be cell-type specific, with the protein capable of moving in every cell-type so far tested. Moreover, our results demonstrate that VP22 can move between different cell-types. An additional advantage of VP22 import is that normal endocytosis is not involved, and VP22 can gain rapid access to the cell. Other potential exogenous delivery systems, such as Tat (Mann and Frankel, 1991) or bacterial toxins (Stenmark et al., 1991; Prior et al., 1992), are taken up by endocytosis, which may result in the retention of these proteins for lengthy periods in endosomal vesicles, and thus excluded from the intracellular environment, or more drastically delivered to lysosomes and degraded. Finally, the conflicting requirements in viral-mediated delivery, to limit replication and pathogenesis while introducing the protein of interest efficiently to many cells, are of relevance to the consideration of delivery protocols. A debilitated virus such as the gH$^-$ virus used here would provide an excellent biological system for delivering a vector into a limited number of cells from which VP22, or fusion proteins of VP22, would then spread out into many cells in a tissue, ensuring widespread delivery.

Experimental Procedures

Cells and Viruses

COS-1, Vero, and Hela cells were all maintained in Dulbecco's modified minimal essential medium containing 10% newborn calf serum. BHK-21 cells were maintained in Glasgow modified minimal essential medium containing 10% newborn calf serum and 10% tryptose phosphate broth. The HSV-1 disabled gH$^-$ virus strain utilized in this study was kindly provided by T. Minson. Infections with this virus were carried out at a multiplicity of 0.1, using a virus stock that had been propagated on a complementing gH-expressing cell line. Following 1 hr adsorption, the medium was replaced with Dulbecco's minimal essential medium containing 2% calf serum, and infection allowed to progress for 30 hr prior to fixation.

Plasmids

The eukaryotic expression vector pGE109 contains the VP22 open reading frame under the control of the human cytomegalovirus IE promoter, and has been described previously (Elliott and Meredith, 1992). The expression vectors for the C-terminal deletion mutant of VP22, plasmid Δ267, and the VP22-peptide fusion protein, pUL49ep (Leslie et al., 1996), were generous gifts from J. McLauchlan. The HSV-1 glycoprotein L expression vector was kindly provided by T. Minson. The expression vector for β-galactosidase, pBAG, has been described previously (Price et al., 1987). The GFP expression vector, pEGFPN1 was obtained from Cambridge Biosciences. Plasmid pGE150 was constructed by inserting the BamH1 fragment from pUL49ep, containing the entire VP22 open reading frame, into the BamH1 site of pEGFPN1, resulting in a fusion between VP22 and GFP.

Antibodies and Reagents

The monoclonal anti-VP22 antibody P43 and the polyclonal anti-gL antibody were kindly provided by D. Meredith and T. Minson, respectively. The polyclonal antibody AGV30 was raised against a GST-VP22 fusion protein. Its specificity was tested by Western blotting prior to use in this study. Monoclonal anti-T antigen pAB419 has been described previously (Harlow et al., 1981). Monoclonal antibodies to γ-adaptin and α-tubulin were obtained from Sigma, and the monoclonal antibody to β-galactosidase was obtained from Promega. The polyclonal anti-rGFP antibody was obtained from Cambridge Biosciences.

Brefeldin A (used at 2.5 μg/ml), nocodazole (used at 10 μg/ml), and cytochalasin D (used at 1 μM) were obtained from Sigma. Texas Red-conjugated transferrin (used at 50 μg/ml) was obtained from Molecular Probes.

Transfection and Microinjection

Cells for immunofluorescence were plated into 6-well trays (6 × 35 mm) at a density of 2×10^5 cells per well, containing one coverslip per well. They were transfected with a DNA mix consisting of 200 ng of expression plasmid made up to 2 μg with pUC19 DNA, using the calcium phosphate precipitation technique modified with BES [*N,N*-bis(2-hydroxyl)-2-aminoethanesulfonic acid]-buffered saline in place of HEPES-buffered saline.

To carry out a cell import assay, cell-free extracts were made from 10^6 transfected COS-1 cells in a buffer consisting of 10 mM HEPES (pH 7.9), 400 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT and 5% glycerol. Half of the extract was then added to the medium covering 5×10^5 cells in a 35 mm well, containing a single coverslip.

Nuclear fractions were prepared from cells exposed to these soluble extracts by washing cells twice in cold PBS, followed by incubation on ice for 10 min in 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM and 1% Triton X-100. Nuclei were pelleted by a brief centrifugation, washed once in the same buffer, and lysed in SDS-PAGE loading buffer.

For microinjection, cells were plated into 6-well trays at a density of 5×10^5 cells per well, containing one coverslip per well. Cells were injected with a solution containing 100 ng/μl plasmid DNA in PBS, using a Carl Zeiss semiautomatic microinjector in manual mode.

Immunofluorescence and Microscopy

Cells to be processed for immunofluorescence were washed with PBS and fixed for 15 min at room temperature with either 100% methanol for cell permeabilization, or 4% paraformaldehyde in PBS for cell-surface staining. The samples were then blocked with 10% calf serum in PBS for 15 min at room temperature. Primary antibody was added in the same solution and incubated for 20 min at room temperature. Following 2 × 5 min washes with PBS, secondary antibodies were added in the blocking solution and incubated for 10 min. Two additional 5 min washes were carried out before the coverslips were mounted in glycerol. To stain DNA, propidium iodide was added to the glycerol mountant at a final concentration of 3 μg/ml. Samples were examined in dual channels using a Bio-Rad MRC600 confocal microscope, and images processed using Adobe Photoshop software.

Western Blot Analysis

Proteins were separated by SDS-PAGE, and the gels transferred to nitrocellulose filters and reacted with the appropriate primary antibody. A horseradish peroxidase–linked secondary conjugate was utilized, and reactive bands visualized using the Enhanced Chemiluminescence (ECL) Detection Reagents (Amersham).

Acknowledgments

We thank John McLauchlan for generously providing plasmids pU-L49ep and Δ267; Tony Minson and Helena Browne for the gH⁻ virus, the gL expression plasmid, and anti-gL antibody; and David Meredith for antibody P43. We also thank Jerry Mouzakitis for the characterization of antibody AGV30. We are grateful to Kate Nobes for her assistance with the microinjection experiments and to Lynn Enquist for comments on the manuscript. This work was funded by Marie Curie Cancer Care.

Received October 3, 1996; revised November 26, 1996.

References

Baldin, V., Roman, A.-V., Bosc-Bierne, I., Amalric, F., and Bouche, G. (1990). Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. EMBO J. *9*, 1511–1517.

Blaho, J.A., Mitchell, C., and Roizman, B. (1994). An amino acid sequence shared by the herpes simplex virus 1 alpha regulatory proteins 0, 4, 22, and 27 predicts the nucleotidylylation of the UL21, UL31, UL47, and UL49 gene products. J. Biol. Chem. *269*, 17401–17410.

Cao, Y., and Pettersson, R.F. (1990). Human acidic fibroblast growth factor overexpressed in insect cells is not secreted into the medium. Growth Factors *3*, 1–13.

Cao, Y., Ekstrom, M., and Pettersson, R.F. (1993). Characterisation of the nuclear translocation of acidic fibroblast growth factor. J. Cell. Sci. *104*, 77–87.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., and Prasher, D. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802–805.

Dargan, D. (1986). The structure and assembly of herpes viruses. In Viral Structure, J.R. Harris and R.W. Horne, eds. (New York: Academic Press), pp. 359–437.

DeMarchi, F., d'Adda di Fagagna, F., Falaschi, A., and Giacca, M. (1996). Activation of transcription factor NF-κB by the Tat protein of human immunodeficiency virus type 1. J. Virol. *70*, 4427–4437.

Derossi, D., Joliot, A.H., Chassaings, G., and Prochiantz, A. (1994). The third helix of the antennapedia homeodomain translocates through biological membranes. J. Biol. Chem. *269*, 10444–10450.

Elliott, G.D., and Meredith, D.M. (1992). The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. J. Gen. Virol. *73*, 723–726.

Elliott, G., Mouzakitis, G., and O'Hare, P. (1995). VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. J. Virol. *69*, 7932–7941.

Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R.A., Wingfield, P., and Gallo, R.C. (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J. Virol. *67*, 277–287.

Fawell, S., Seery, J., Daikh, Y., Moore, C., Cheng, L.L., Pepinsky, B., and Barsoum, J. (1994). Tat-mediated delivery of heterologous proteins into cells. Proc. Natl. Acad. Sci. USA *91*, 664–668.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J. Virol. *66*, 341–348.

Fuller, A.O., Santos, R.E., and Spear, P.G. (1989). Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit attachment to cells but prevent penetration. J. Virol. *63*, 3435–3443.

Gompels, U., and Minson, A.C. (1986). The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology *153*, 230–247.

Harlow, E., Crawford, L.V., Pim, D.C., and Williamson, N.M. (1981). Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. *39*, 861–869.

Helland, D.E., Welles, J.L., Caputo, A., and Haseltine, W.A. (1991). Transcellular transactivation by the human immunodeficiency virus type1 *tat* protein. J. Virol. *65*, 4547–4549.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A.C., and Johnson, D.C. (1992). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. *66*, 2240–2250.

Jackson, A., Friedman, S., Zhan, X., Engleka, K.A., Forough, R., and Maciag, T. (1992). Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. Proc. Natl. Acad. Sci. USA *89*, 10691–10695.

Joliot, A.H., Pernelle, C., Deagostini-bazin, H., and Prochintz, A. (1991). Antennapedia homeobox peptide regulates neural morphogenesis. Proc. Nat. Acad. Sci. USA *88*, 1864–1868.

Krasney, P.A., and Young, P.R. (1992). Further aspects of IL-1 beta secretion revealed by transfected monkey kidney cells. Cytokine *4*, 134–143.

Kuchler, K. (1993). Unusual routes of protein secretion: the easy way out. Trends Cell Biol. *3*, 421–426.

Leslie, J., Rixon, F.J., and McLauchlan, J. (1996). Overexpression of the herpes simpex virus type 1 tegument protein VP22 increases its incorporation into virus particles. Virology *220*, 60–68.

Lippincott Schwartz, J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.P., Yuan, L.C., and Klausner, R.D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell *60*, 821–836.

Mann, D.A., and Frankel, A.D. (1991). Endocytosis and targeting of exogenous HIV-1 Tat protein. EMBO J. *10*, 1733–1739.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.-M., and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell *84*, 923–932.

Pastan, I.H., and Willingham, M.C. (1981). Journey to the centre of the cell: role of the receptosome. Science *214*, 504–509.

Price, J., Turner, D., and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus mediated gene transfer. Proc. Natl. Acad. Sci. USA *84*, 156–160.

Prior, T.I., Fitzgerald, D.J., and Pastan, I. (1992). Translocation mediated by domain II of pseudomonas exotoxin A: transport of barnase into the cytosol. Biochemistry *31*, 3555–3563.

Robinson, M., and Kries, T. (1992). Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of Brefeldin A and G protein activators. Cell *69*, 129–138.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55–63.

Rothman, J.E., and Wieland, F.T. (1996). Protein sorting by transport vesicles. Science *272*, 227–234.

Rubartelli, A., and Sitia, R. (1995). Entry of exogenous polypeptides in to the nucleus of living cells: facts and speculation. Trends Cell Biol. *5*, 409–412.

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. EMBO J. *9*, 1503–1510.

Stenmark, H., Moskaug, J., Madshus, I.H., Sandvig, K., and Olsnes, S. (1991). Peptides fused to the amino-terminal end of diphtheria toxin are translocated to the cytosol. J. Cell. Biol. *113*, 1025–1032.