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SFV infection in CHO cells: cell-type specific restrictions to productive virus entry at the cell surface

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SUMMARY

Alphaviruses, such as Semliki Forest virus, normally enter cells by penetration from acidic organelles of the endocytic pathway. The virions are internalised intact from the cell surface before undergoing acid-induced fusion in endosomes. To investigate the possibility that endocytosis might play a role in delivering virions to specific sites for replication, we compared SFV infection of baby hamster kidney (BHK) cells and Chinese hamster ovary (CHO) cells following either normal virus fusion in endosomes or experimentally-induced fusion at the cell surface. Whereas baby hamster kidney cells were infected efficiently following fusion in endosomes or at the plasma membrane, Chinese hamster ovary cells were only infected following fusion from endocytic organelles. Virions fused at the plasma membrane of CHO cells failed to initiate viral RNA and protein synthesis. Similar results were observed when CHO cells were challenged with a rhabdovirus, vesicular stomatitis virus.

These data suggest that in certain cell types a barrier, other than the plasma membrane, can prevent infection by alpha- and rhabdoviruses fused at the cell surface. Moreover, they suggest the endocytic pathway provides a mechanism for bringing viral particles to a site, or sites, in the cell where replication can proceed.

Key words: Semliki Forest virus, Virus entry, Membrane fusion

INTRODUCTION

Enveloped viruses gain access to their host cells through membrane fusion (Marsh and Helenius, 1989; White, 1992). For many of these viruses, including alpha-, orthomyxo- and rhabdo-viruses, fusion only occurs when the virions are exposed to mildly acidic pH (reviewed by Marsh and Helenius, 1989). These acidic conditions are encountered in endocytic organelles following endocytosis of intact virions (Marsh et al., 1983). The low pH triggers conformational changes in the viral envelope proteins that activate the fusion machinery of the viruses (Doms et al., 1985; Kielian and Helenius, 1985; Stegmann et al., 1990; Salminen et al., 1992; Wahlberg et al., 1992; Bullough et al., 1994; Klimjack et al., 1994). The strict pH dependence prevents virions fusing with the plasma membrane under normal conditions. Furthermore, fusion and infection can be blocked by agents that raise the pH of acidic intracellular organelles (Helenius et al., 1980, 1982; Marsh et al., 1982).

In contrast, some other enveloped viruses, including the paramyxoviruses (see White et al., 1983), many retroviruses (McClure et al., 1990) and the primate immunodeficiency viruses (HIV-1 and HIV-2) (Stein et al., 1987; McClure et al., 1988), are not dependent on acidic conditions for entry and, for Sendai virus at least, appear to undergo membrane fusion with similar efficiencies at both acid and alkaline pH (White et al., 1983). For these viruses fusion can potentially occur at the cell surface or within acidic organelles following endocytosis and both routes may be used (Pudney and Song, 1994). For HIV-1, however, productive infection can occur by fusion at the cell surface without the need for endocytosis (Maddon et al., 1988; Orloff et al., 1991; Pelchen-Matthews et al., 1995).

In experiments on the alphavirus Semliki Forest virus (SFV), we found that normal entry into baby hamster kidney (BHK-21) cells could be blocked by weak bases or carboxylic ionophores that raise the pH of acid organelles. However, this block to infection could be circumvented by acidifying the medium to artificially induce viruses to fuse with the plasma membrane (Helenius et al., 1980, 1982; Marsh et al., 1982). This suggested that cells can be productively infected by both the plasma membrane and the endocytic routes, and that the choice as to which route is used is determined solely by the pH-dependence of the viral fusion reaction.

Here we have studied the entry of SFV into Chinese hamster ovary (CHO) cells. Whereas endocytosis of SFV in these cells leads to productive infection, viruses fused to the plasma membrane do not infect. The results suggest that cell specific restrictions may influence the routes adopted by viruses to enter cells, and that for certain viruses barriers other than the plasma membrane can prevent productive infection via the cell surface.

MATERIALS AND METHODS

Reagents

All tissue culture reagents were from Gibco Ltd (Paisley, Scotland),

and other chemicals were from Sigma (Poole, England), unless indicated otherwise. Tissue culture plastic was from Falcon, and radioactive reagents were from Amersham International plc (Little Chalfont, England).

Cells and viruses

Cell lines were maintained as follows: baby hamster kidney cells (BHK-21; obtained from EMBL, Heidelberg) were grown in Glasgow's modified minimal essential medium (GMEM) supplemented with 10% tryptose phosphate broth (TPB), 5% foetal calf serum (FCS), 2 mM glutamine, 100 U.ml^{-1} penicillin and 0.1 mg.ml^{-1} streptomycin (PenStrep); Chinese hamster ovary cells (CHO-wtB; obtained from I. Mellman, Yale University) were grown in alpha MEM supplemented with 5% FCS, glutamine and PenStrep.

Semliki Forest virus (SFV) was grown in BHK-21 cells as described (Marsh and Helenius, 1980). Radiolabelled SFV was prepared by growing SFV infected BHK-21 cells in methionine-free DMEM supplemented with [^{35}S]methionine, or DMEM containing [^{3}H]uridine, and was purified by sucrose gradient centrifugation. Pyrene-labelled SFV was prepared as described (Bron et al., 1993; Stegmann et al., 1993). BHK cells (5×10^7 per roller bottle) were grown for 48 hours in GMEM containing 10 μg.ml^{-1} pyrene hexadecanoic acid (Molecular Probes; Eugene, Oregon). Prior to infection the label was removed. The cells were washed with BM (Binding medium: RPMI-1640, without bicarbonate, containing 0.2% BSA, 10 mM Hepes, 10 mM MES, and adjusted to pH 6.8) and subsequently incubated with SFV at 10 pfu/cell in BM. After 90 minutes at 37°C, GMEM containing 10% FCS was added and the incubation continued for 16 hours. The virus was collected and purified by sucrose density gradient centrifugation (Marsh and Helenius, 1980). Viral phospholipid was determined after extraction of the lipids (Bligh and Dyer, 1959), by phosphate analysis (Böttcher et al., 1961). Viral protein was determined according to the method of Peterson (1977).

Vesicular Stomatitis virus (VSV-Indiana strain; obtained from Prof. R. Weiss, Institute of Cancer Research, London) was grown in BHK-21 cells essentially as described (Matlin et al., 1982). A 90 mm plate of confluent BHK-21 cells (1×10^7 cells) was infected with plaque purified wild-type VSV at an m.o.i. of 0.1 in 3 ml G-MEM containing 1% FCS, 10% TPB, PenStrep and glutamine. After 1 hour a further 9 ml of medium was added and the cells incubated at 37°C. After 18 hours the supernatant was removed and clarified by centrifugation (3,000 rpm for 10 minutes). The titre of virus was estimated by plaque assay on BHK-21 cells.

Viruses were snap frozen in aliquots in liquid nitrogen and stored at –80°C.

Infectivity assays

[^{3}H]Uridine incorporation

SFV and VSV infection were assayed by [^{3}H]uridine incorporation into viral RNA as described (Helenius et al., 1982). Cells, grown to confluence on 13 mm glass coverslips in 24-well trays, were infected with SFV or VSV as indicated for individual experiments. Two hours after infection the medium was removed and replaced with 0.25 ml of the normal growth medium (see above) containing 2 mg.ml^{-1} actinomycin D, 20 mM NH_4Cl and 1 μCi [^{3}H]uridine. The cells were labelled for 1-2 hours at 37°C in a CO_2 incubator. Subsequently the [^{3}H]uridine medium was removed and the cells washed with 2 ml ice-cold PBS containing 10 mM uridine (PBS-U). The coverslips were transferred to a new 24-well plate, washed again with PBS-U and left on ice. After 15 minutes the PBS-U was removed and replaced with 0.5 ml 10% trichloroacetic acid (TCA) and left on ice for at least 1 hour. Finally, the coverslips were drained and transferred to scintillation vials for radioactivity determinations.

Biochemical assays

Binding, endocytosis and uncoating were measured as described (Marsh and Helenius, 1980; Helenius et al., 1982). Radiolabelled SFV was bound to cell monolayers in 35 mm dishes in 0°C BM. To internalise bound virus the cells were warmed to 37°C, by addition of prewarmed BM, and incubated at 37°C for the required time. Subsequently, the cells were returned to ice and washed with 4°C BM. Virus fusion at the cell surface was induced by incubating the cells in 37°C, pH 5.5, BM for 1 minute. Subsequently, the cells were washed with 0°C pH 6.8 BM and placed on ice. To measure total cell-associated virus the cells were scraped from the plates and collected by centrifugation. To distinguish between cell surface-bound and internalised virus, cells were washed once with PBS containing 6.8 mM Ca^{2+} and 5.0 mM Mg^{2+} (PBS^{++}) and incubated with 0.5 mg.ml^{-1} Proteinase K (Boehringer Mannheim, Lewes, England) in PBS^{2+} for 1 hour at 4°C on a platform shaker. The cells were transferred to centrifuge tubes, washed twice with BM, resuspended in 0.1 ml PBS and transferred to vials containing 5 ml Packard 'Emulsifier-Safe' liquid scintillation fluid for scintillation counting.

Immunofluorescence

Cells (50% confluent) on glass coverslips were infected with SFV as described above. Four hours after infection the cells were fixed with 2% paraformaldehyde in PBS^{++} for 10 minutes, quenched with 50 mM NH_4Cl, and permeabilised with 0.1% Triton X-100 for 4 minutes at room temperature. The cells were stained with affinity-purified rabbit anti-SFV envelope glycoprotein antibodies for 1 hour, followed by rhodamine-conjugated goat anti-rabbit antibodies (Pierce and Warriner, Chester, England) and mounted in Moviol. Cells were viewed on a Nikon Optiphot-2 microscope equipped with an MRC Bio-Rad 1024 laser scanning attachment. Images were recorded using identical gain, background, and zoom settings, and subsequently transferred to a Macintosh computer. Images were assembled using Adobe Photoshop and transferred to T-Max100 ASA film through a Management Graphics slide recorder.

Lipid-mixing assays

Confluent BHK and CHO cells on 35 mm Petri dishes were incubated with pyrene-labeled SFV (10 or 20 nmol viral phospholipid) in BM for 90 minutes at 4°C. Unbound virus was removed by 3 washes with BM. Endocytosis was induced by incubating the cells in 37°C BM for 30 minutes. Alternatively, cells with bound virus were exposed to 37°C BM adjusted to pH 5.6 for 2 minutes to induce fusion at the plasma membrane. To inhibit endosomal acidification 10 μM monensin or 20 mM NH_4Cl was included in the medium during virus binding and during the 37°C incubations. After endocytosis or low pH treatment, the cells were placed on ice and the medium replaced by 2 ml ice-cold Hanks/Hepes buffer, pH 7.4, containing 50 mM NH_4Cl to prevent any further fusion. Subsequently the cells were scraped with a disposable cell scraper (Costar) and transferred to a fluorimeter cuvette. Pyrene fluorescence emission spectra were monitored at 37°C from 350 nm to 550 nm in a Shimadzu RF5001 PC spectrofluorimeter. Excitation was at 343 nm. Data were processed and spectra were corrected for cell backgrounds using the manufacturer's software. Total cell-associated virus was quantified by determining the monomer fluorescence in the samples at 378 nm after the addition of Triton X-100 to 0.5% (v/v). The extent of fusion (f) was determined using the formula $f = 100\ (E_0\text{-}E)/E_0$, where E_0 and E represent the excimer fluorescence at 480 nm at time zero and after experimental treatment, respectively (Bron et al., 1993). Fluorescence was converted to viral particles by assuming 30,000 phospholipid molecules per virion.

RESULTS

SFV entry into cells is strictly dependent on exposure of the virions to low pH (Helenius et al., 1980, 1982; White and Helenius, 1980). Thus infection usually requires that virions

bind to cell surface receptors, undergo endocytosis and penetrate from acidic endosomes (Marsh et al., 1983; Helenius, 1984). In BHK-21 cells the endocytosis step in SFV entry can be by-passed by exposing cells, with bound virus, to pH 5.5 medium - below the pH threshold for fusion (Helenius et al., 1980; White et al., 1980). An incubation of 1 minute at this pH is sufficient to induce fusion with the plasma membrane and infection of the cell. Furthermore, fusion at the cell surface will permit infection of BHK cells in the presence of agents such as ammonium chloride or monensin that raise the pH of acidic organelles and inhibit entry through endosomes (Helenius et al., 1982; Marsh et al., 1982).

CHO cells are not infected following fusion of SFV at the cell surface

To investigate whether fusion induced at the plasma membrane permits infection in cells other than BHK, we attempted to infect CHO cells with SFV by the endocytic route or by fusion at the cell surface. To monitor infection, cells were fixed 4 hours after treatment with virus, permeabilized and stained with specific antibodies to detect newly synthesised SFV envelope glycoproteins. The bright staining seen in Fig. 1 'Control' panels shows that BHK and CHO cells were infected with SFV through the endocytic route. No staining of BHK cells or CHO cells was observed when infection was carried out in the presence of monensin. When BHK cells with bound virus were treated with pH 5.5 medium for 1 minute to induce fusion at the plasma membrane, bright staining for the viral envelope glycoprotein was subsequently seen in most cells. This indicated that fusion of virions at the plasma membrane led to productive infection. Similar results were obtained if ammonium chloride was included in the medium instead of monensin (not shown). By contrast, CHO cells treated with an identical cell surface fusion protocol showed very few stained cells. This suggested that either viruses failed to fuse at the surface of CHO cells following low pH treatment, or that virions fused at the cell surface do not initiate infection in these cell.

CHO cells fail to synthesise viral RNA following SFV fusion at the cell surface

The observation that CHO cells could be infected through the endocytic pathway (Fig. 1) indicated that these cells were capable of the binding, endocytosis and intracellular fusion of SFV, and of the subsequent synthesis of the viral structural proteins. Control experiments indicated that CHO and BHK cells bound radiolabelled SFV with similar kinetics and to similar levels (not shown). To determine whether the lack of viral protein synthesis following low pH treatment of SFV-bound CHO cells was due to a failure of CHO cells to replicate the viral RNA, we analysed cells for viral RNA synthesis following infection through the normal endocytic route or through fusion at the cell surface. SFV was bound to BHK or CHO cells by incubation at 4°C in medium with or without 20 mM NH_4Cl. Subsequently, the cells were warmed to 37°C in normal medium, or medium adjusted to pH 5.5, with or without NH_4Cl. The pH 5.5 medium was removed from the cells after 1 minute and replaced with normal medium with or without NH_4Cl. Two hours later the cells were labelled with [^{3}H]uridine.

Fig. 2 shows that in the presence of actinomycin D little [^{3}H]uridine is incorporated in the absence of virus infection in

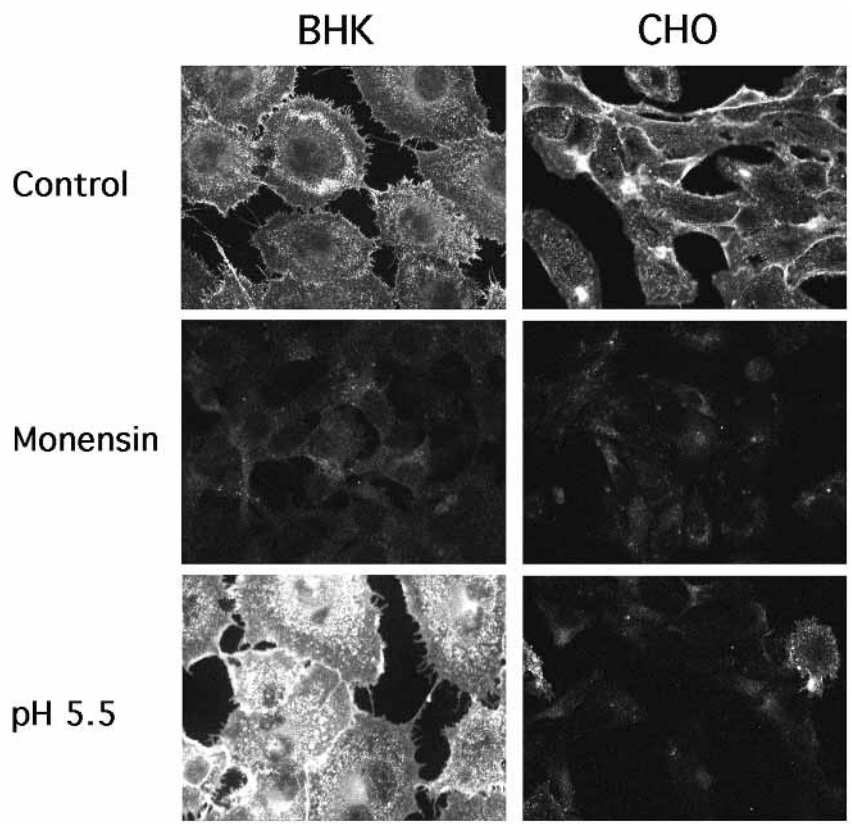

Fig. 1. Immunofluorescence analysis of SFV infection in BHK-21 and CHO cells. BHK-21 (left hand panels) and CHO (right hand panels) were incubated with 10 pfu/cell SFV for 30 minutes at 4°C, in the presence (Monensin and pH5.5) or absence (Control) of 10 μM monensin. Unbound virus was washed away and the cells warmed to 37°C in binding medium (Control), binding medium containing 10 μM monensin (Monensin), or binding medium containing 10 μM monensin and adjusted to pH 5.5 (pH 5.5). The medium was replaced after 1 minute with pH 6.8 binding medium (Control) or pH 6.8 binding medium containing 10 μM monensin (Monensin and pH 5.5) and the cells incubated for 2 hours at 37°C. Subsequently all coverslips were transferred to normal culture medium and incubated for a further 2 hours at 37°C before staining.

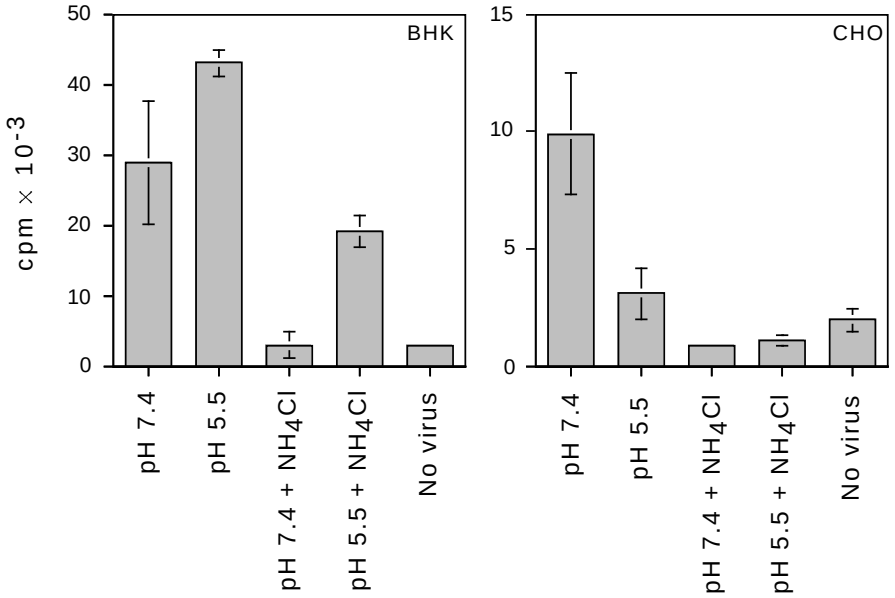

Fig. 2. Viral RNA synthesis in BHK-21 and CHO cells following exposure to SFV. BHK-21 and CHO cells were incubated with SFV (100 pfu/cell) in BM or BM containing 20 mM NH_4Cl for 30 minutes at 4°C. A relatively high titre was used to ensure the majority of cells bound virus during the incubation. Subsequently, the free virus was washed away and the cells warmed to 37°C in BM with or without 20 mM NH_4Cl. After 2 hours the medium in each well was replaced with 1 ml DMEM containing 1 μCi [^{3}H]uridine and actinomycin D. The cells were incubated for a further 2 hours at 37°C before measuring the [^{3}H]uridine incorporation. The means and s.d. of quadruplicate wells for a representative experiment are shown.

both BHK and CHO cells ('No virus' control). However, when cells were infected with SFV through the endocytic route incorporation of approximately 32×10^3 CPM per BHK cell coverslip was recorded. Incorporation was enhanced slightly if cells were incubated in pH 5.5 medium. Inclusion of NH_4Cl during the infection reduced incorporation to background levels, but even in the presence of NH_4Cl a brief acid treatment of cells with bound virus returned incorporation and infection to 60% of control levels. These results support the morphological data above, and previous experiments (Helenius et al., 1982), indicating that viruses fused to the cell surface can productively infect BHK-21 cells.

When the same experiment was performed on CHO cells, infection through the normal endocytic route resulted in viral RNA synthesis as judged by [^{3}H]uridine incorporation. However, when CHO cells with bound virus were treated with low pH medium in the absence of NH_4Cl there was a 70% reduction in the level of [^{3}H]uridine incorporation. In these samples the endocytic pathway was still functional for entry. If the endocytic pathway was inhibited by inclusion of 20 mM NH_4Cl during the binding and entry stages, background levels of [^{3}H]uridine incorporation were measured indicating that viral entry was efficiently inhibited. When NH_4Cl treated cells were briefly exposed to low pH medium, conditions which allowed efficient infection of BHK-21 cells, only a slight increase over background [^{3}H]uridine incorporation was measured.

Together these results indicate that, in contrast to BHK cells, brief exposure of CHO cells with bound SFV to acid medium does not allow efficient infection. Similar results were obtained

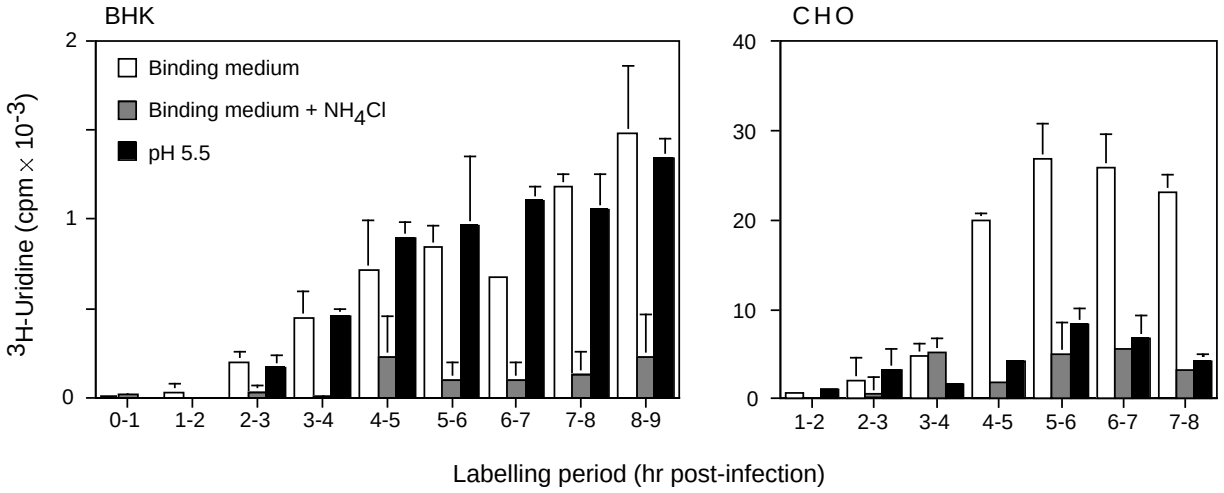

Fig. 3. Time course of viral RNA synthesis in BHK-21 and CHO cells. BHK-21 and CHO cells were incubated with 100 pfu/cell SFV at 4°C for 30 minutes. Free virus was washed away and the cells warmed to 37°C in BM to allow infection through the endocytic route (□), or in BM plus 20 mM NH_4Cl to block infection through the endocytic route (■). A third set of coverslips were incubated for 1 minute in BM adjusted to pH 5.5 to induce fusion of bound virions at the plasma membrane and were then returned to BM containing 20 mM NH_4Cl (■). At the indicated times pairs of coverslips for each set of conditions were transferred to medium containing [^{3}H]uridine for 60 minutes at 37°C.

if monensin was used instead of NH_4Cl. In control experiments, cells treated with low pH medium in the absence of virus were unaffected in their ability to incorporate [^{3}H]uridine or [^{35}S]methionine (not shown) indicating that the brief treatment at low pH does not affect the cellular RNA or protein synthesis machineries.

Kinetics of SFV/RNA synthesis in BHK-21 and CHO cells

In the previous experiments the synthesis of viral RNA was measured at a single time period 2-4 hours after viral infection. To determine whether the onset of RNA synthesis might occur more slowly in CHO cells than in BHK-21 cells, we repeated the experiment and measured [^{3}H]uridine incorporation during 1 hour pulses over a 7-9 hour time period.

As indicated in Fig. 3, [^{3}H]uridine incorporation was first observed 2 hours after initiating infection of BHK-21 cells through the endocytic route. Incorporation increased over the time of the experiment and was maximal 8-9 hours after the addition of virus to cells. RNA synthesis was reduced when NH_4Cl was included in the medium, consistent with the ability of weak bases to inhibit entry through the endocytic pathway. However, a similar time course and magnitude of infection were seen when virus was fused at the cell surface in the presence of NH_4Cl.

When [^{3}H]uridine incorporation was measured over the same time course in CHO cells, SFV RNA synthesis began 2 and 3 hours after the addition of virus to cells and reached a peak 3-4 hours later. As with the BHK cells, NH_4Cl inhibited infection through the endocytic route. When virus was fused at the cell surface only low levels of uridine incorporation were seen across the course of the experiment. Furthermore, there was no indication that the onset of RNA synthesis occurred later in CHO cells. Together these data indicate that viral infection and RNA synthesis has similar kinetics in both BHK and CHO cells and that the lack of infection seen when viruses were fused at the plasma membrane was not due to slower replication kinetics in the CHO cells.

Low pH-induced fusion of SFV on CHO cells

SFV fusion has been characterised extensively using both liposomes and cellular membranes. For wild-type SFV the conformational changes in the envelope protein complex required for fusion are initiated when the pH is lowered to about 6.2 with maximum conformational change and fusion activity occurring in the range pH 5.8-6.2 (White and Helenius, 1980; White et al., 1980; Kielian and Helenius, 1985). Furthermore, the fusion reaction is rapid, essentially being complete within 60 seconds of changing the pH (White and Helenius, 1980; White et al., 1980; Kielian and Helenius, 1985; Bron et al., 1993). To ensure the block to CHO cell infection following low pH treatment of bound virions is not due to a failure to undergo fusion, we analysed SFV fusion at the cell surface using protease resistance assays. For these experiments [^{35}S]methionine-labelled SFV was bound to CHO cells at 4°C. Subsequently, the cells were warmed to 37°C in pH 6.6 or pH 5.5 medium for 1 minute. The cells were then returned to 4°C and virions remaining at the cell surface were removed by treatment with Proteinase K. The counts that remained cell-associated following protease treatment represent virus that has

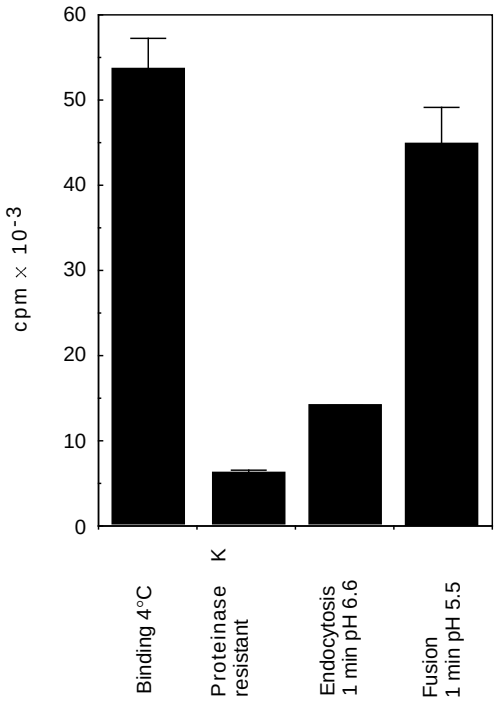

Fig. 4. Low pH-induced cell surface fusion of SFV with CHO cells. CHO cells were incubated with [^{35}S]methionine labelled SFV for 1 hour at 4°C. The free virus was washed away and the cells then either kept on ice in BM (Binding and Proteinase K resistant), or incubated for 1 minute at 37°C in pH 6.6 BM (Endocytosis) or pH 5.5 BM (Fusion). With the exception of the 'Binding' samples, the cells were then incubated with Proteinase K for 1 hour at 4°C. Subsequently, the cells from each plate were collected and the cell-associated radioactivity determined. The mean of duplicate plates are shown.

either undergone endocytosis (pH 6.6) or a combination of endocytosis and cell surface fusion (pH 5.5) during the 37°C incubation.

Without warm-up Proteinase K removed approximately 90% of the bound radioactivity from the cell surface (Fig. 4). If cells were warmed in pH 6.6 medium for 1 minute, 28% of the bound radioactivity became protease resistant. As pH 6.6 is above the pH required for fusion, this indicates the amount of virus taken up by endocytosis during the 1 minute incubation at 37°C. When cells were warmed to 37°C at pH 5.5, approximately 85% of the pre-bound radioactivity became protease resistant. As the endocytotic pathway can also operate at 37°C this suggests that a minimum of 50% of the cell surface virus undergoes fusion during the 1 minute incubation at 37°C. The proportion of virus that fuses may be higher as low pH medium is known to inhibit endocytosis (Davoust et al., 1987; Sandvig et al., 1987). In similar experiments using [^{3}H]uridine-labelled virus, in which the label is restricted to the viral RNA, 65% of the bound virus became protease resistant following low pH treatment (data not shown). Similar results were previously reported for SFV fusion at the surface of BHK-21 cells (White et al., 1980; Kielian et al., 1984). Thus by the criteria of protease resistance, SFV fuses with the plasma membrane of CHO cells when the pH is lowered appropriately.

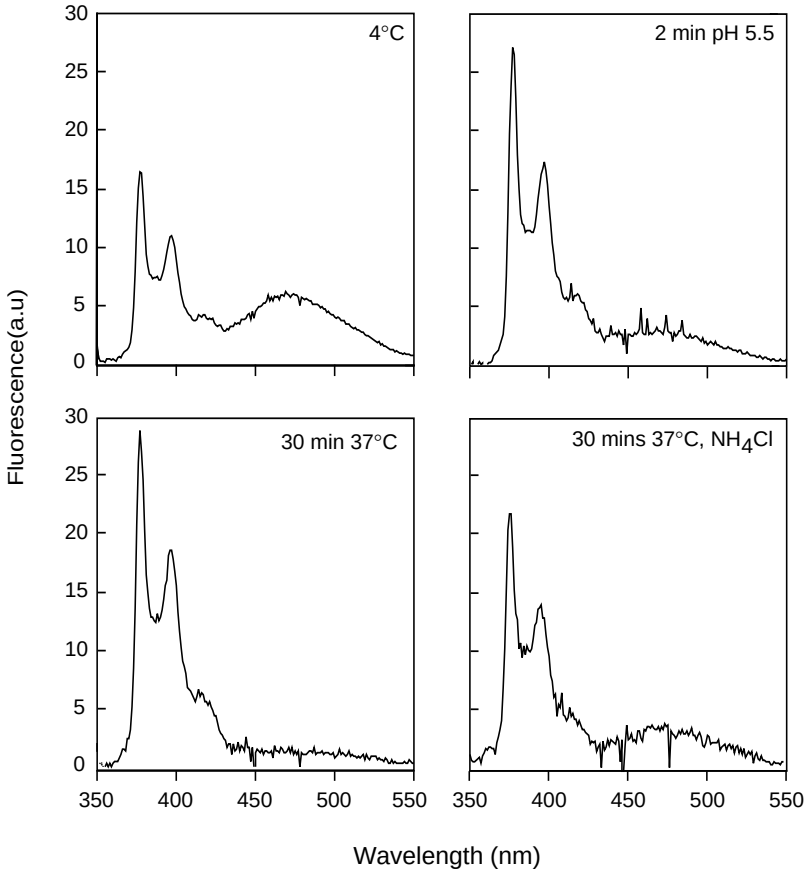

Fig. 5. Fusion of pyrene-labelled SFV on CHO cells. CHO cells were incubated with pyrene-labelled SFV for 90 minutes at 4°C. Subsequently, the cells were either kept on ice (4°C), or were warmed to 37°C for 30 minutes in the presence (30 minutes, 37°C, NH_4Cl) or absence (30 minutes, 37°C) of 20 mM NH_4Cl. To induce fusion at the cell surface, cells were incubated in pH 5.5 BM for 2 minutes (2 minutes, pH 5.5). Subsequently the cells were placed on ice and the medium replaced with ice-cold Hanks/Hepes buffer, pH 7.4, containing 50 mM NH_4Cl. The cells were transferred to a fluorimeter cuvette and pyrene fluorescence emission spectra monitored at 37°C from 350 nm to 550 nm. Excitation was at 343 nm. The fluorescence intensities are indicated in arbitary units.

Cell surface fusion activity is equivalent on both BHK-21 and CHO cells

To ensure that the low pH-induced association of radiolabelled SFV with CHO cells was due to fusion of the virus with the cell membrane, and not some non-fusogenic change in the virus that reduced its susceptibility to removal by Proteinase K, we assayed fusion directly using fluorescent lipids. Pyrene-labelled SFV was bound to confluent plates of BHK or CHO cells for 90 minutes at 4°C. The free virus was washed away and the cells subsequently warmed to 37°C either in medium at neutral pH or in medium adjusted to pH 5.6. Where indicated NH_4Cl was added to inhibit endosomal acidification. Subsequently the cells were scraped into medium containing 50 mM NH_4Cl to inhibit any further fusion and transferred to a spectrofluorimeter cuvette.

Fusion activity was assessed by monitoring a shift in the ratio of pyrene excimers to monomers following dilution of the lipid probe in cellular membranes (Stegmann et al., 1993). A representative experiment is illustrated in Fig. 5. Virus bound to CHO cells (4°C) shows a prominent peak at about 470 nm corresponding to pyrene excimers in the membrane of labelled virus. When cells with bound virus were incubated at 37°C for 30 minutes and the fluorescence emission spectrum recorded, the peak of excimer fluorescence was diminished and a corresponding increase in the monomer peaks at 380 and 400 nm was seen (30 minutes 37°C). Inclusion of 20 mM NH_4Cl during the 37°C incubation inhibited the excimer to monomer shift (30 minutes 37°C, NH_4Cl). When cells with bound virus were incubated briefly at pH 5.5 for 2 minutes at 37°C in the presence of 20 mM NH_4Cl a shift of the excimer fluorescence into the monomer peak was also observed (pH 5.5). Similar results were observed in BHK cells (data not shown). When the fusion activity was calculated from the excimer fluorescence, we estimated that approximately 40 and 80% of the cell-associated viruses underwent fusion with BHK and CHO cells, respectively, during uptake of virus through the endocytic route (Fig. 6). The difference in efficiency of virus fusion in these two cell lines may reflect the faster rates of SFV endocytosis observed in CHO cells (data not shown). Fusion was inhibited in both cell lines by NH_4Cl. Significantly, approximately 50% of cell-associated virus underwent fusion when both BHK cells and CHO cells were incubated in low pH-medium, indicating that both cell types were able to support low pH-induced viral fusion at the cell surface to similar extents. Furthermore, the extent of fusion seen with the lipid mixing assay was close to that measured by protease resistance. Similar results were obtained when monensin was used instead of NH_4Cl. Together these data indicate that the block to SFV infection of CHO cells via fusion at the cell surface occurs at a step post fusion.

Other viruses

To investigate whether viruses other than SFV also failed to infect CHO cells following fusion at the cell surface, we tested CHO and BHK cells with the rhabdovirus vesicular stomatitis virus (VSV). Infection was assayed by [^{3}H]uridine incorporation into viral RNA as previously documented. Although the level of incorporation was generally lower than that seen for SFV, we again found that VSV could infect both BHK and

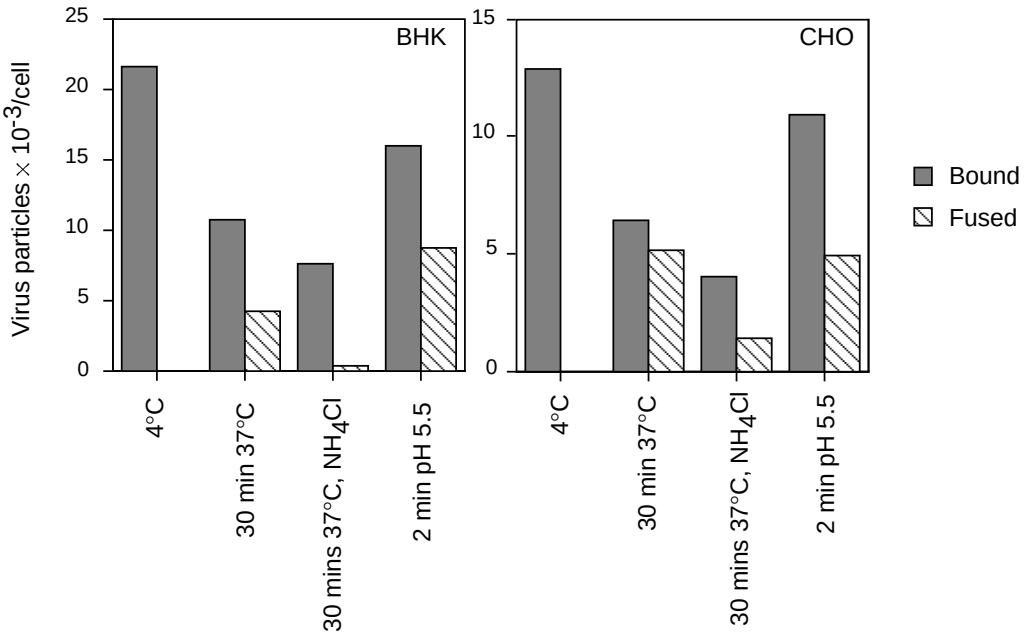

Fig. 6. Fusion activity of pyrene-labelled SFV on BHK and CHO cells. The data from Fig. 5, and from a parallel experiment with BHK-21 cells (not shown), were used to calculate the fusion activity from the excimer fluorescence. Total cell-associated virus was quantified by determining the (monomer) fluorescence in the samples at 378 nm after the addition of Triton X-100 to 0.5% (v/v).

CHO cells through the endocytic pathway (Fig. 7). However, only BHK cells were infected following incubation of cells with bound virus in low pH-medium.

DISCUSSION

The mechanism of SFV entry into cells is one of the best understood of any viral entry pathway (Kielian and Jungerwirth, 1990). Numerous experiments have established that this virus binds to receptors on the cell surface, is internalised through clathrin-coated vesicles and delivered to endosomes (Fries and Helenius, 1979; Helenius et al., 1980; Marsh and Helenius, 1980; Marsh et al., 1983; Kielian and Helenius, 1985; Salminen et al., 1992; Wahlberg et al., 1992). In endosomes the low pH environment triggers a conformational change in the viral envelope glycoproteins that leads to fusion of the virus and endosome membrane and injection of the nucleocapsid into the cytoplasm (Helenius et al., 1980, 1982; Marsh et al., 1983). Subsequently, the nucleocapsid is disassembled and synthesis of the viral replication machinery and RNA is initiated on the cytoplasmic aspect of endosomal and lysosomal membranes (Froshauer et al., 1988).

The necessity for SFV to be exposed to low pH imposes the strict requirement for endocytosis. It has, therefore, been assumed that the prime reason these viruses undergo the potentially hazardous journey into the endocytic pathway is to find an appropriate environment to trigger fusion. Indeed experiments with BHK cells indicate that the requirement for endocytosis can be by-passed if virions are fused with the plasma

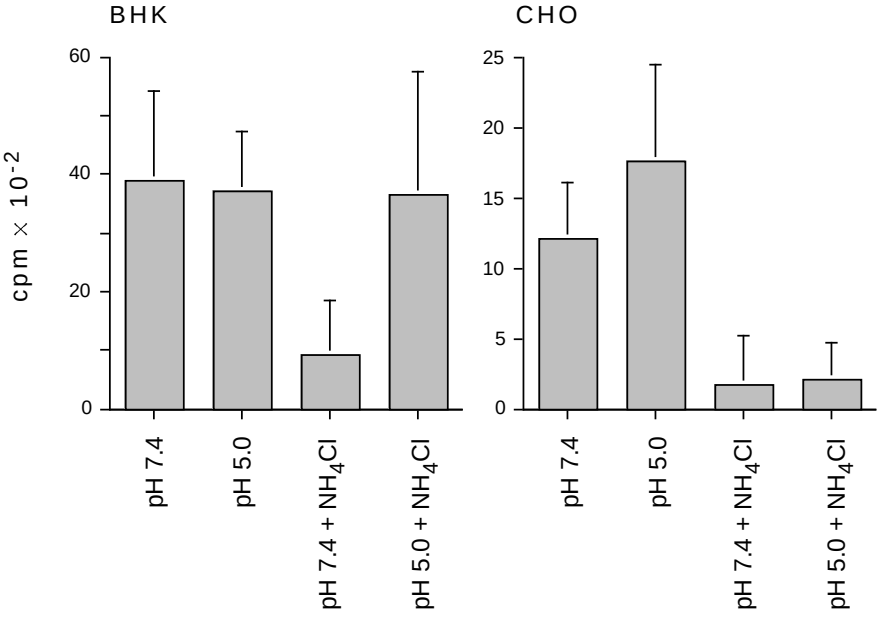

Fig. 7. VSV infection in BHK-21 and CHO cells. BHK-21 and CHO cells were incubated with VSV (approx. 30 pfu/cell) for 30 minutes at 4°C. Free virus was washed away and the cells subsequently warmed to 37°C in BM with or without 20 mM NH_4Cl. Parallel wells of cells were warmed to 37°C in pH 5.0 BM for 1 minute and then transferred to medium at pH 7.4 with or without 20 mM NH_4Cl. After 2 hours the medium in each well was replaced with 1 ml DMEM containing 1 μCi [^{3}H]uridine and incubated for a further two hours at 37°C. The means and s.d. of quadruplicate wells for a representative experiment are shown.

membrane by transiently lowering the pH of the culture medium (Helenius et al., 1980, 1982). However, the experiments described here indicate that SFV fusion at the cell surface does not lead to productive infection in all cell types and suggest that endocytosis might play additional roles in facilitating productive infection.

The data show that SFV can efficiently infect both BHK and CHO cells through the endocytic route and that this infection can be inhibited by weak bases and carboxylic ionophores that neutralise the endosomal environment. Whereas infection of BHK cells in the presence of weak bases and carboxylic ionophores can be rescued by acid-induced fusion of virions at the cell surface, infection of CHO cells by this route is inefficient. Low pH-induced fusion of SFV with the plasma membrane was demonstrated directly using both protease resistance and lipid mixing assays and was found to be equally efficient for CHO and BHK cells in both assays. Thus the inability to infect CHO cells at the cell surface was not due to a failure to undergo fusion. Instead, in CHO cells there is a post-fusion block to infection following penetration at the plasma membrane that is not present when virions enter the cell through endocytic organelles.

In this study we have focussed on CHO cells and SFV. However, experiments with VSV indicate that the block is not restricted to SFV and that the effect is determined by the properties of the cell rather than those of the virus. Circumstantial evidence in the literature indicate that other acid-dependent viruses also fail to infect certain cells when induced to fuse at the cell surface. Thus VSV and the avian influenza virus fowl plague virus fail to infect MDCK cells through the apical plasma membrane when incubated at low pH (Matlin et al., 1981, 1982). VSV fails to infect Vero cells (T. Kreis, personal communication), and monocytes are resistant to the flavivirus West Nile virus (Gollins and Porterfield, 1986), after exposure of surface bound virions to low pH medium. Furthermore, the observation that fusion at the cell surface does not lead to productive infection is not unique to CHO cells, but is also seen with MDCK, Vero, monocytes and, in our hands, NIH3T3 murine fibroblasts and hybridoma cells challenged with either VSV (Exley, 1989) or SFV (data not shown). In contrast, normal rat kidney cells and HeLa cells, at least, behave similarly to BHK and are permissive for SFV and VSV infection following cell surface fusion.

The post-fusion events that lead to the onset of SFV replication are relatively well characterised. Following fusion, interaction of 60 S ribosomal RNA with the SFV capsid protein facilitates uncoating of the nucleocapsid and the initiation of protein synthesis from the viral positive sense RNA genome (Singh and Helenius, 1992; Wengler et al., 1992). Thus delivery of the viral nucleocapsid to the cytoplasm and access to ribosomes is sufficient to initiate infection. SFV nucleocapsids delivered through the plasma membrane of CHO cells may fail to undergo these uncoating events or, alternatively, a step in the process leading to viral RNA synthesis may be inhibited. At present the nature of this putative post-fusion block is unclear. We favour the notion that elements of the cortical cytoskeleton might, at least in part, be responsible. This structure is highly developed in many cells and comprises actin filaments and a host of associated proteins that support and regulate the activities of the plasma membrane (Bray et al., 1986). In lymphoid cells in particular, the cortex can be in the order of 100 nm or more thick and can be seen to exclude cytoplasmic organelles including ribosomes from the environment adjacent to the plasma membrane (Bray et al., 1986; Vitale et al., 1995). Presumably any virus that fuses with the plasma membrane requires a mechanism to bring its nucleocapsid or genetic material through this cortex in order to infect the cell. How this translocation occurs is unclear. Viruses might have means to locally destabilise the cortex, for example the HIV protease is reported to cleave actin and several actin-binding proteins (Shoeman et al., 1991, 1993; Tomasselli et al., 1991; Adams et al., 1992). Alternatively, viruses may interact with the cytoskeletal proteins that make up the cortex and exploit the properties of these proteins to penetrate the cell (Cudmore et al., 1995; Hottiger et al., 1995). However, the pH-dependent viruses that require endocytosis appear to capitalise on the properties of endocytic vesicles to get around this putative barrier. Endocytic vesicles, in particular clathrin coated vesicles, are continually budding from the plasma membrane and delivering their cargo to endosomes. In so doing these vesicles must also traverse the cortex. Again the mechanism through which endocytic vesicles make this crossing is unknown, however, interactions with microfilaments and/or microtubules might play a role (Gottlieb et al., 1993; Jin and Snider, 1993; Jackman et al., 1994; Durrbach et al., 1996).

Whether the cortex is responsible for the block to cell surface infection of CHO cells by SFV is unclear. Morphological experiments show a visible cortical structure beneath the plasma membrane of CHO cells. A similar structure is not as obvious in our BHK cells, but we cannot exclude the possibility that differential sensitivities to fixation and preparation for electron microscopy might account for these differences. Attempts to break the block to infection using cytochalasins have not been successful. However, although these agents induce marked changes in the distribution of actin associated with stress fibres their effects on the cortical cytoskeleton are not well characterised. Nevertheless, it is clear from our analysis with CHO cells that fusion at the cell surface may not be sufficient to allow productive infection of the cell and that additional constraints on virus entry exist. How these constraints apply to other viruses, in particular pH-independent viruses that are frequently believed to undergo fusion or penetration at the cell surface, remains to be established.

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