Lipid-raft-dependent Coxsackievirus B4 internalization and rapid targeting to the Golgi

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Abstract

Coxsackievirus B4 (CBV4), a member of the Picornavirus genus, has long been implicated in the development of insulin-dependent diabetes mellitus (IDDM), by viral-induced pancreatic cell damage. Although the pancreotropic nature of this virus is well documented, the early stages of CBV4 viral infection that involve the attachment of virions to the cell surface by binding to their cellular receptors followed by entry into the cell, are poorly understood. In this study, we show that the entry of CBV4 requires functional lipid rafts as the site of virus attack. In addition, we show that this virus is endocytosed independently of clathrin-associated machinery and is delivered to the Golgi via a lipid-raft-dependent mechanism.

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Keywords: Lipid raft; Coxsackievirus B4; Golgi apparatus

Introduction

Insulin-dependent diabetes mellitus (IDDM), also known as type I diabetes, is caused by progressive destruction of pancreatic β cells. Epidemiological studies have shown that Coxsackievirus infections are associated with the development of type I diabetes (Banatilava, 1987; Horwitz, 1990; Yoon et al., 1979). The most commonly detected strain in diabetic and prediabetic patients is Coxsackievirus B4 (Horwitz, 1990). This virus infects several tissues, but primarily it causes degenerate destruction of large regions of exocrine pancreas as well as β islets leading to IDDM.

Although the pancreotropic nature of this virus is well documented, very little is known about the pathways by which this virus infects the pancreatic cells of the host. Understanding the transport mechanism of CBV4 into pancreatic cells is essential for successful development of efficacious prophylactic and therapeutic measures.

Both the attachment and entry of any virus into a host cell are dependent on the interaction of the virus with the plasma membrane. Although the plasma membrane was envisioned as a fluid randomly arranged lipid bilayer, recent advances have demonstrated that this important cellular barrier is more sophisticated. Several studies have shown the existence of cholesterol-enriched microdomains that are essential for cellular function (Damjanovitch et al., 1999; Edidin, 2001; Jacobson and Dietrich, 1999; Jacobson et al., 1995). Lipid rafts, or microdomains as they are also called, appear as detergent/insoluble/resistant glycolipid-enriched membrane domains and are believed to perform diverse functions by providing a specialized microenvironment in which the relevant molecules for the specific biological processes are partitioned from the rest of the plasma membrane and are concentrated into the raft (Horejsi et al., 1999).

Such partitioning has been shown to play a role in a plethora of biological functions such as signal transduction, membrane transport, T cell activation, and host–pathogen interactions (Romagnoli and Bron, 1997; Viola et al., 1999; Xavier et al., 1998). Lipid rafts seem to play a role in the internalization of bacterial lipopolysaccharide (Triantafilou et al., 2002b) as well as budding of HIV (Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 2001) and influenza virus (Scheiffele et al., 1999), while the involvement of rafts in their entry has also been postulated (Dimitrov, 2000). Recent studies have also shown that several enteroviruses including echovirus 1 (Marjomaki et al., 2002), echovirus 11 (Stuart et al., 2002), as well as **
Coxsackievirus A9 (Triantafilou and Triantafilou, 2003) utilize lipid rafts for cell entry.

In this study, we set out to determine the initial steps of CBV4 infection of pancreatic cells, which include the interaction of CBV4 with the plasma membrane as well as subsequent internalization and transport within the cell. There are several endocytic pathways that viruses utilize. For example, Semliki Forest virus enters the cell through clathrin-mediated endocytosis (Helenius et al., 1980), while Simian virus 40, a simple nonenveloped DNA virus that replicates in the nucleus, enters cells through caveolae (Hummeler et al., 1970). After binding to the cell surface via MHC class I molecules, SV40 moves laterally along the plasma membrane until trapped in caveolae (Pelkmans et al., 2001), whereas Echovirus 11 clusters the GPI-anchored protein CD55 leading to lipid-raft-dependent internalization (Stuart et al., 2002).

We investigated CBV4 interactions with its cellular receptor Coxsackie Adenovirus receptor protein (CAR) (Bergelson et al., 1997) as well as with CD55 which is utilized by several Coxsackie B viruses (CBV1, CBV3, CBV5) as a receptor molecule (Shafran et al., 1995) for virus binding. Using fluorescent imaging techniques, we followed the intracellular interactions of the virus. We found that, initially, CBV4 is colocalized with CD55 and Coxsackie-adenovirus-receptor protein (CAR) within lipid rafts. Subsequently, we found that CBV4 is internalized and rapidly reaches the Golgi apparatus independently of clathrin-interacting endocytic machinery. This transport pathway appears to be raft specific, yet not all CBV4 receptors follow it as there is a percentage of CAR visualized within endosomes, thus suggesting that CAR can also follow the clathrin endocytic machinery. Overall, our data suggest that this virus takes advantage of the considerable fluidity of lipid rafts between the plasma membrane and the Golgi complex to gain access to the cell. Our findings agree with Nichols et al. (2001c), who have demonstrated a novel rapid recycling pathway from the plasma membrane to the Golgi that is followed by lipid raft markers. It seems that CBV4 attachment induces the recruitment of CAR and CD55 into membrane microdomains, and takes advantage of the internalization of these domains to reach and accumulate in the Golgi apparatus.

Results

Cell surface interactions of CBV4: FRET imaging of lipid rafts

The concentration of CBV4 particles and receptor molecules involved in CBV4 attachment within lipid rafts was examined using fluorescence resonance energy transfer (FRET). Lipid rafts tend to aggregate into distinct patches on the cell membrane that can be visualized by fluorescence microscopy using Cy3-conjugated cholera toxin, which binds to GM-1 ganglioside, a raft-associated lipid.

We measured FRET in terms of dequenching of donor fluorescence after complete photobleaching of the acceptor fluorophore. Increased donor fluorescence after complete destruction of the acceptor indicated that the donor fluorescence was quenched in the presence of the acceptor because of energy transfer. We tested the energy transfer efficiency in our system using as a positive control the energy transfer from MAbs Cy3–W6/32 and Cy5-MCA1115 to two different epitopes on MHC-class I molecules (Table 1), which showed that the maximum energy transfer efficiency (E) was 39 ± 1.7% (under idealized FRET conditions, a maximum E of 40.96% is achieved). A negative control involving irrelevant surface receptors such as the transferrin receptor and integrin αvβ3 was performed by testing the energy transfer between Cy3-M073401 specific for the transferrin receptor and Cy5-MCA757G specific for the integrin αvβ3, the resulting E was 2 ± 1.5%.

To visualize whether CBV4 viruses were localized in lipid rafts, we measured FRET on pancreatic cells between CBV4 particles (using Cy3-specific CBV4 Fab) and GM1 ganglioside, a raft-associated lipid (using Cy5-cholera toxin). Dequenching was observed once the Cy5 was photo-bleached (E = 19 ± 1.0%), suggesting that the virus concentrates in the lipid rafts of the plasma membrane (Fig. 1). Control experiments were performed with the Cy5-cholera toxin using Cy3-specific CBV4 Fab without the presence of CBV4 particles. The results showed that the energy transfer efficiency was 1.8 ± 0.5%, thus verifying that there was no nonspecific interaction. We also tested the association of transferrin receptor (Cy3-M073401), which is a known nonraft marker with Cy5-cholera toxin for GM1-ganglioside. Our data showed an energy transfer efficiency of 2.2 ± 0.6%, thus verifying that the transferrin receptor is not concentrated in lipid rafts (Table 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Energy transfer efficiency values between donor–acceptor pairs</th>
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<tr>
<td><strong>Before virus infection</strong></td>
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</tr>
<tr>
<td>MHC-class I</td>
<td>β2-m</td>
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<tr>
<td>Transferrin receptor</td>
<td>Integrin αvβ3</td>
</tr>
<tr>
<td>CBV4-E2</td>
<td>GM1 ganglioside</td>
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<tr>
<td>CD55</td>
<td>GM1 ganglioside</td>
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<tr>
<td>CAR</td>
<td>GM1 ganglioside</td>
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<tr>
<td>Transferrin receptor</td>
<td>GM1 ganglioside</td>
</tr>
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</table>

| **After CBV4-E2 infection** | | |
| CD55 | GM1 ganglioside | 30 ± 1.2 |
| CAR | GM1 ganglioside | 29 ± 0.5 |
| Transferrin receptor | GM1 ganglioside | 2.6 ± 0.8 |

Energy transfer between different pairs was detected from the increase in donor fluorescence after acceptor photobleaching. Data represent mean ± standard deviation of three independent experiments.
We similarly examined whether CD55 and CAR protein localize in lipid rafts before CBV4 infection, by using Cy3-MCA914 Fab specific for CD55 and Cy3–C1079 specific for CAR protein, respectively, and Cy5-cholera toxin for the GM1 ganglioside. Our results showed that there was FRET observed between CD55 and GM1 ganglioside (23%); thus, CD55 was present in lipid rafts before CBV4 infection. On the other hand, there was a small percentage of CAR protein associating with lipid rafts, giving FRET values of 11% (Table 1).

We then examined whether CD55 or CAR molecules were recruited into the raft after infection with CBV4. FRET experiments were therefore performed with CD55 and GM1 ganglioside as well as CAR protein and GM1 ganglioside in the presence of CBV4 particles. We found that there was an increased association between CD55 (Cy3-MCA914 Fab) and GM1 ganglioside (Cy5-cholera toxin) (E = 30 ± 1.2%) as well as CAR and GM1 ganglioside (E = 29 ± 0.5%) in the presence of CBV4 particles (see Table 1). Our data show that upon virus infection, although CD55 is localized in lipid rafts (which is expected as it is a GPI linked protein), there is a significant increase of CD55 molecules in the lipid rafts after virus infection. There is also a significant increase of CAR molecules recruited in the raft upon CBV4 infection. However, after virus infection, there was no association between transferrin receptor and GM1-ganglioside (E = 2.6 ± 0.8%). To rule out the possibility that the FRET observed was due to random distribution, we varied the ratio of donors and acceptors used to label the proteins of interest (Fig. 1). E was found to be independent on acceptor density, to be sensitive to donor/acceptor ratio, and not to go to zero at low surface density, thus suggesting that the FRET values observed were due to clustered molecules and not random associations.

**Entry route**

To investigate the entry route of CBV4 into pancreatic cells, we used vital stains for known vesicular compartments in pancreatic cells as well as fluorescent probes for CBV4 (using a Cy5-specific CBV4 Fab), CD55 (TRITC-MCA914 Fab), and CAR (TRITC-C1079).
CBV4 does not concentrate in lysosomes

Lysosomes are acidic vesicles rich in hydrolytic enzymes and represent the site of degradation of extracellular macromolecules internalized by pinocytosis or phagocytosis. We determined if CBV4 or its receptors CD55, CAR are internalized into lysosomes using a green fluorescent, freely permeant probe with a high selectivity for acidic organelles, Lysotraker Green DND-99 (Loike et al., 1991). Pancreatic cells were incubated for different time periods (5, 15, 30, and 60 min) with CBV4 particles in the presence of Lysotraker Green DND-99. Triple labeling with Lysotraker Green DND-99, CBV4-specific probe, and CD55-specific probe (TRITC-MCA914 Fab) or CAR-specific probe (TRITC-C1079) was performed. Our results showed that CAR was internalized within lysosomes, while CD55 did not colocalize within lysosomes. The compartment containing internalized CBV4 was readily distinguishable from lysosomes (Fig. 2). Overall, these results suggest that although lysosomes handle the endocytosis of CAR receptors, the vesicular transport of CBV4 from the plasma membrane does not appear to deliver the virus particles to lysosomes.

CBV4 does not concentrate in endosomes

Endosomes are a structurally diverse population of vacuoles and tubules serving as sorting intermediates along both the biosynthetic and endocytic pathways. To determine the CBV4 endocytic pathway, we used a FITC-conjugated EEA1-specific serum to label early endosomes because EEA1 is an early endosome marker. Triple labeling in pancreatic cells with EEA1-specific serum conjugated to FITC (FITC-anti EEA1), CBV4, and CD55 or CAR-specific fluorescent probes at 5, 15, 30, and 60 min postinfection showed no colocalization between the virus and EEA1 or CD55 and EEA1, suggesting that the virus does not enter early endosomes (Fig. 3). However, there was some degree of colocalization between CAR and EEA1 (Fig. 4).

Fluorescent dextran, a hydrophylic polysaccharide with poly-(α-D-1,6-Glucose) linkages, is rapidly taken up by an endocytic process and moves to early and late endosomes (Berlin and Oliver, 2003). We studied the internalization of a fluid-phase marker (FITC-dextran) CBV4 and CD55 or CAR in pancreatic cells. At 30 min p.i., FITC–dextran appeared in a heterogeneous assortment of internalized

Fig. 2. Intracellular distribution of CBV4, CAR, or CD55 and organelle marker for lysosomes at 30-min postinfection. CBV4 particles labeled with Cy5-specific CBV4 Fab are seen as blue. Lysosomes are labeled green with Lysotraker green DND-99. CAR molecules labeled with TRITC-C1079 and CD55 molecules labeled with TRITC-MCA914 are seen as red. A merged image of the three (CBV4, CAR, lysosomes) is shown as well as a merged image of the three (CBV4, CD55, lysosomes). Although CBV4 and CD55 do not overlap with the labeling pattern of lysosomes, there is colocalization between CAR and lysosomes (seen as yellow). Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
vesicles. CBV4 was concentrated in a compartment distinct from FITC–dextran, showing us that the virus does not localize in late endosomes. Neither was CD55, while there was partial colocalization of CAR with dextran as manifested by the punctate yellow staining in the merged images (Fig. 5).

Inhibition of acidification of endosomes

Virions internalized by endocytosis penetrate the cytoplasm by fusing with membranes of endosomes or lysosomes in a pH-independent or -dependent manner. Viruses such as Semliki forest virus (Garoff et al., 1994), Influenza virus (March and Pelschen-Mathews, 2000), and Human Herpesvirus 8 (Akula et al., 2003) require exposure to low pH environment to enter endosomes, while viruses such as duck hepatitis virus enter via pH-independent endocytosis (March and Pelschen-Mathews, 2000). Although we had already seen that CBV4 does not penetrate lysosomes or endosomes, we decided to examine whether a change in pH could affect the virus entry mechanism. We used NH₄Cl, which is a weak lysosomotropic base that diffuses into acidic endosomes, where it becomes protonated. Once protonated, it is unable to diffuse out, thus increasing the pH. The cells were treated with 25 or 50 nM NH₄Cl; the early endosomes were stained with FITC-EEA1-specific serum and we looked at the distribution of CBV4, CAR, or CD55. Our results showed that there was no change in the virus or the CD55 distribution (Fig. 3) while NH₄Cl inhibited CAR entry in early endosomes (Fig. 4).

CBV4 does not concentrate in the ER

Endoplasmic reticulum is the largest endomembrane system within eukaryotic cells and performs a wide variety of functions, including calcium uptake and release, lipid and protein synthesis, protein translocation and folding, glycosylation, concentration and export to the Golgi complex. We used anticalnexin antibody conjugated to FITC (FITC anticalnexin) to label the ER. The infected cells showed no accumulation of virus in the ER. Fluorescent virus was observed in vesicular structures distinct from the ER (Fig. 6).

CBV4 concentrates in the Golgi

To determine whether CBV4 was localized in the Golgi, we used two different markers to label compartments of the Golgi apparatus. Anti-TGN38-specific antibody conjugated to FITC to label the TGN-38 integral protein.
membrane of the trans-Golgi complex (Stephens and Banting, 1999) and the anti GM130 specific antibody to a cis-Golgi matrix protein of the cis-Golgi apparatus structure, which we had conjugated to FITC. Triple labeling in pancreatic cells with FITC-labeled anti-TGN-38 or FITC–anti GM130, CBV4, and CD55 or CAR-specific fluorescent probes at 15, 30, and 60 min p.i. showed colocalization of the virus, CAR, and CD55 with GM130 after 30 to 60 min p.i. (Figs. 7 and 8). No colocalization of CBV4, CD55, or CAR with TGN38 was observed (data not shown), suggesting that the virus and its receptors are mainly concentrated in the cis-Golgi and not the trans-Golgi network.

Effects of clathrin route inhibitors on CBV4 entry

To determine whether CBV4 utilizes clathrin-coated pits for internalization, we used different clathrin inhibitors before CBV4 infection. We utilized various chemicals that we had tested and were not cytotoxic to the cells, such as nocodazole (20 μM) and chlorpromazine (25 μM). Nocodazole is known to block endosomal traffic between early and late endosomes (Gruenberg et al., 1989), whereas chlorpromazine causes the disappearance of clathrin-coated pits from the cell membrane (Joki-Korpela et al., 2001; Wang et al., 1993).

Nocodazole or chlorpromazine did not prevent the efficiency of viral replication, which was analyzed by counting the number of virus-infected immunofluorescence-positive cells (Table 2). Using triple fluorescent labeling, we investigated the concentration of CBV4 as well as CAR and CD55 in the Golgi apparatus after treatment with either of the two chemicals. We found that neither nocodazole nor chlorpromazine prevented the localization of CBV4 and CD55 with the Golgi, nor CBV4 infectivity (Fig. 9). In contrast, both chemicals seem to affect (Fig. 10) but not completely inhibit the localization of CAR. Overall, this data show that virus entry into pancreatic cell is clathrin independent.

Effects of raft-disrupting drugs on CBV4 entry

Because we had already found that CBV4 and its receptor molecules localize in lipid rafts in the initial steps of virus
attachment, we investigated whether lipid raft-disrupting drugs such as nystatin (Anderson et al., 1996) or filipin (Keller and Simons, 1998), which had been tested and were not cytotoxic at the optimal concentrations of 25 μM and 1 μg/ml, respectively, could inhibit viral entry. Using confocal microscopy, we counted the virus-infected immunofluorescence-positive cells (see Materials and methods). Our results showed that cells pretreated with nystatin or filipin showed inhibition of virus infection (Table 2), while fluorescent labeling revealed that treatment with either filipin (data not shown) or nystatin prevented CBV4 colocalization to the Golgi (Figs. 9 and 10), leading us to believe that CBV4 viral entry and targeting to the Golgi apparatus is dependent on lipid raft integrity. The cells’ viability was not affected either

Fig. 5. Intracellular distribution of CBV4, CAR, or CD55 and organelle marker for endosomes. CBV4 particles labeled with Cy5-specific CBV4 Fab are seen as blue. Endosomes are revealed as green with FITC–Dextran. CAR molecules labeled with TRITC-C1079 and CD55 molecules labeled with TRITC-MCA914 are seen as red. A merged image of the three (CBV4, CAR, endosomes) and (CBV4, CD55, endosomes) is shown. Although CBV4 particles do not overlap with the labeling pattern of endosomes, there is a partial overlap between CAR and endosomes, which is seen as yellow. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Intracellular distribution of CBV4, CAR, and anticalnexin antibody conjugated to FITC (FITC anticalnexin) to label ER at 60-min postinfection. CBV4 particles labeled with Cy5-specific CBV4 Fab are seen as blue. ER is seen as green with FITC anti calnexin. CAR molecules labeled with TRITC-C1079 are seen as red. A merged image of the three is also shown. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
by nystatin or filipin treatment as the drug-treated cells excluded trypan blue.

Discussion

For pancreatic cells to be infected by CBV4, the virus has to first attach to the cell surface using specific receptors, such as CAR (Bergelson et al., 1997), subsequently penetrate the plasma membrane, and traffic through the cell to target its RNA to the nucleus. In this study, we investigated the attachment as well as the entry route of CBV4 in pancreatic cells.

Initially, we looked at the distribution on the plasma membrane of CBV4 virions, CAR (Bergelson et al., 1997), the main Coxsackievirus B receptor, as well as CD55, which is a protein utilized by several Coxsackieviruses for binding (Shafren et al., 1995).

Using FRET, we found that CBV4 virus particles as well as both CAR and CD55 are concentrated within lipid rafts.
on the plasma membrane during the attachment stage of the CBV4 infectious cycle. CD55, which is a GPI-linked protein, seems to be constitutively localized in lipid rafts, whereas CAR although present in a smaller percentage than CD55 in the raft, was shown to increasingly accumulate in the raft upon the presence of CBV4 virions.

Following the attachment of the virus, we attempted to track the entry route of the virus within the cell. Several nonenveloped viruses have been shown to use the clathrin-endocytic machinery to gain access into the cells. Examples of such viruses include canine parvovirus, adenovirus, parechovirus 1, rhinovirus 14, as well as polyomaviruses (DeTulleo and Kirchhausen, 1998; Joki-Korpela et al., 2001; Pho et al., 2000). Other viruses, such as Simian virus 40, which binds to MHC-class I, utilizes caveolar endocytosis (Hummeler et al., 1970).

To elucidate CBV4 entry, we utilized triple-labeling fluorescent imaging to visualize CBV4, CAR, or CD55, with markers for different intracellular compartments. We found that CBV4 did not colocalize with lysosomes, endo-

![Fig. 8. Intracellular distribution of CBV4, CAR, and FITC–anti-GM130 for cis-Golgi at 0, 15, 30, and 60-min postinfection is shown. CBV4 particles labeled with Cy5-specific CBV4 Fab are seen as blue. cis-Golgi is seen as green. CAR molecules labeled with TRITC-C1079 are seen as red. Merged images of the three are also shown (CBV4, Golgi, CAR), which show that CAR and CBV4 concentrate in the cis-Golgi (colocalization of all three; CBV4, Golgi, CAR is seen as white) at 30 and 60 min. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
somes, or ER over different time periods (0, 15, 30, or 60 min p.i.). In contrast, it was found that CBV4 was rapidly targeted to the Golgi apparatus and specifically to the *cis*-Golgi network 30–60 min p.i.

In previous studies it has been shown that CD55 is not necessary for virus entry (Shafren et al., 1995), but since CD55 is a GPI-linked protein and thus constitutively resides in the lipid raft, it targets the Golgi through a clathrin-independent route, similar to CBV4.

On the other hand, although CAR, a transmembrane protein, is localized in lipid rafts, it seems to be able to follow a clathrin-dependent route, as we have visualized CAR in lysosomes and endosomes. However, to a smaller percentage, it also follows a clathrin-independent route, because it cannot be completely inhibited in all cells by anticalathrin inhibitors, thus demonstrating the capability of this protein to use both routes, which could explain why adenovirus, which uses CAR as its main receptor, has the ability to enter cells via two different endocytic processes (Asbourne Excoffon et al., 2003).

In an attempt to verify whether CBV4 entry was dependent on clathrin-coated pits, we utilized different clathrin inhibitors before incubation with CBV4 virions. Chemicals such as nocodazole and chlorpromazine, which are known to block endosomal traffic between early and late endosomes (Gruenberg et al., 1989), and to cause the disappearance of clathrin-coated pits from the cell membrane (Wang et al., 1993), respectively, were also utilized. These chemicals did not inhibit CBV4 internalization. Furthermore, using triple labeling, we visualized

<table>
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<tr>
<th>Chlorpromazine (µM)</th>
<th>% Infectivity</th>
<th>% Toxicity</th>
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<tr>
<td>0</td>
<td>72.5</td>
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<td>25</td>
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<th>Nocodazole (µM)</th>
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<td>0</td>
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<td>71.8</td>
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<tr>
<th>Filipin (µg)</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>1</td>
<td>11.8</td>
<td>3.5</td>
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Toxicity was determined by trypan blue staining. This table represents data from three independent experiments.

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**Table 2**

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<thead>
<tr>
<th>Percentage of CBV4 infected PANC-1 cells (2 h p.i.) in cells treated with different drug inhibitors</th>
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<tbody>
<tr>
<td>% Infectivity</td>
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<tr>
<td>----------------</td>
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<tr>
<td>Chlorpromazine (µM)</td>
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<tr>
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<td>Nystatin (µM)</td>
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<tr>
<td>Filipin (µg)</td>
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**Fig. 9.** Intracellular distribution of CBV4, CD55, and FITC–anti-GM130 for *cis*-Golgi at 60-min postinfection in the presence of chlorpromazine and nystatin. CBV4 particles labeled with Cy5-specific CBV4 Fab are seen as blue. Golgi is seen as green. CD55 molecules labeled with TRITC-MCA914 are seen as red. A merged image of the three is also shown. Colocalization of all three: CBV4, Golgi, CD55 is seen as white. Scale bar = 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CBV4, CAR, or CD55 and Golgi markers. We found that preincubation with the clathrin-route inhibitors did not affect CBV4 infection of pancreatic cells, thus suggesting that CBV4 gains entrance and is targeted to the cis-Golgi via a clathrin-independent route.

In addition, CBV4 internalization seemed to be lipid raft dependent. In the presence of lipid-raft-disrupting drugs, such as nystatin or filipin, CBV4 infectivity was inhibited. Furthermore, targeting of CBV4 to the Golgi network was inhibited upon disruption of the lipid raft integrity. Our studies suggest that lipid raft formation is crucial for CBV4 entry and trafficking through the pancreatic cell.

Previous observations have shown that the entire cholesterol, ganglioside-rich microdomain or “lipid raft”, where we have shown that CBV4, CAR, and CD55 reside, is constantly shuttling back and forth from the cell surface to the Golgi (Nichols et al., 2001a; Vishwajeet et al., 2001). Our studies confirm this because when we incubated pancreatic cells with Cy3-labeled cholera toxin, which binds to GM-1 ganglioside (a lipid raft marker), the cholera toxin was rapidly targeted from the cell surface to the Golgi apparatus indistinguishably of CBV4, CD55, and CAR (data not shown).

Our data suggests that CBV4 is dragged to the Golgi along with the lipid raft and a small percentage of CAR via a novel clathrin-independent endocytic route. CBV4 seems to have evolved to take advantage of the plasma membrane–Golgi cycling pathway that is followed by sphingolipids, which make up lipid rafts. By following this route, CBV4 gains rapid entrance into the pancreatic cell and reaches its biosynthetic machinery within only 30–60 min p.i. Like Echovirus 1 (Marjomaki et al., 2002), Echovirus 11 (Stuart et al., 2002), and Coxsackievirus A9 (Triantafilou and Triantafilou, 2003), CBV4 has been shown to utilize lipid rafts for cell entry. The pathway that viruses follow upon internalization seems to be dictated by the receptor or the receptor complexes that the virus utilizes. Thus, other viruses that utilize molecules, which reside in lipid rafts, may also follow this rapid internalization pathway from the plasma membrane to the Golgi apparatus.

Fig. 10. Intracellular distribution of CBV4, CAR and FITC–anti-GM130 for cis-Golgi at 60-min postinfection in the presence of nocodazole and nystatin. CBV4 particles labeled with Cy5- specific CBV4 Fab are seen as blue. Golgi is seen as green. CAR molecules labeled with TRITC-C1079 are seen as red. A merged image of the three is also shown. Colocalization of all three; CBV4, Golgi, CAR is seen as white. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Materials and methods

Cell lines

Human pancreatic cell line (PANC-1) was maintained in DMEM with Glutamax and 4500 mg/l glucose medium (Gibco) containing 10% heat-inactivated fetal bovine serum. Human pancreatic cell line (ASPC-1) was maintained in RPMI with Glutamax (Gibco) containing 10% heat-inactivated fetal bovine serum.

Viruses

The diabetogenic strain, CBV4 Edwards strain 2 (E2), was kindly provided by Dr. J.W. Yoon (Yoon et al., 1979). The virus was propagated in PANC-1 cells. For immunofluorescence experiments, viruses were used at an MOI of 10.

Materials

CD55-specific mAb MCA914 was obtained from Serotec (U.K.). RemB CAR-specific mAb for initial experiments was kindly provided by Dr. Bergelson, while CAR-specific mAb C1079 was obtained from Europa Bioproducts. Horse polyclonal serum specific for CBV4 was purchased from American Tissue Type Collection (ATTC). Lysotraker Green DND-99, DiOC6, FITC-Dextran, FITC-Con A, NBDC6 ceramide were obtained from Molecular Probes Inc. EEA1-specific goat polyclonal serum and Calnexin-specific rabbit serum were obtained from Santa Cruz Biotechnology (CA). Mouse anti-GM130 was purchased from Transduction Labs/BD PharMingen (San Diego, CA). Mouse anti-TGN38 was from Affinity Bioreagents (Golden, CO). The antibodies were conjugated to either Cy3 or Cy5 (using Cy3 and Cy5 labeling kits from Amersham-Pharma- cia), or they were conjugated to either FITC or TRITC (Molecular Probes Inc.) according to the manufacturers’ instructions. Cholera toxin was purchased from List Labs. The following chemicals were obtained from Sigma and used to study the viral entry procedures: nocodazole (20 μM) for depolymerization of microtubules; chloropromazine (25 and 50 μM) to inhibit the formation of clathrin coated pits; nystatin (25 μM) and filipin (1 μg/ml) for the disruption of rafts; and NH4Cl (25 and 50 mM), a weak base, to inhibit low-pH-dependent endosomal fusion.

Cell labeling for FRET

PANC-1 or ASPC-1 cells on microchamber culture slides (Lab-tek, Gibco) were labeled with 100 μl of a mixture of donor-conjugated antibody Cy3) and acceptor-conjugated antibody Cy5. The cells were rinsed twice in PBS/0.02% BSA, before fixation with 4% formaldehyde for 15 min. The cells were fixed to prevent potential reorganization of the proteins during the experiment.

Confocal imaging

PANC-1 or ASPC-1 cells were grown on microchamber culture slides. Cells were infected with CBV4-E2; after different times postinfection (p.i), they were exposed to fluorescent stains for organelles (these experiments were done by binding the virus to cells at low temperatures followed by a shift to 37 °C) and subsequently fixed with 4% formaldehyde for 15 min. For virus and receptor staining, fluorescent probes were added and incubated for 60 min in PBS/0.02% BSA/0.02% Saponin, then washed three times with PBS. The slides were mounted on Prolong antifade (Molecular Probes).

When specific inhibitory drugs were used, the chemicals (nocodazole, chloropromazine, nystatin, filipin) were added to the cell culture medium before attachment of the virus for 30 min and they were also present during infection. The efficiency of viral replication was analyzed by counting the number of immunofluorescence-positive cells by confocal microscopy after 1, 2, 4, and 6 h. Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63 × Zeiss objective. The images were analyzed using LSM 2.5 image analysis software (Carl Zeiss, Inc.). FITC and TRITC were detected using the appropriate filter sets. Using typical exposure times for image acquisition (less than 5 s), no fluorescence was observed from a FITC-labeled specimen using the TRITC filters, nor was FITC fluorescence detected using the TRITC filter sets.

FRET measurements

FRET is a noninvasive imaging technique used to determine molecular proximity. FRET can occur over 1–10-nm distances, and effectively increases the resolution of light microscopy to the molecular level. It involves nonradiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor. The rate of energy transfer is inversely proportional to the sixth power of the distance, between donor and acceptor. The efficiency of energy transfer (E) is defined with respect to r and R0, the characteristic Forster distance by:

\[ E = \frac{1}{[1 + (r/R_0)^6]} \]  

In the present study, FRET was measured using a previously described method (Bastiaens and Jovin, 1996; Nich- ols et al., 2001b; Triantafilou et al., 2002a). Briefly, samples were labeled with donor- and acceptor-conjugated antibodies and energy transfer was detected as an increase in donor fluorescence (quenching) after complete photo-bleaching of the acceptor molecule. Cy5 was bleached by continuous excitation with an arc lamp using a Cy5 filter. FRET images were calculated from the increase in donor fluorescence after acceptor photobleaching by E.
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References


