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Enterovirus 70 Enters Hela Cells by a Clathrin and Dynamin Dependent Route

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Enterovirus 70 Enters HeLa Cells by a Clathrin- and Dynamin-Dependent Route

By

Ahmar Khan

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ABSTRACT

Endocytosis, which is normally associated with cellular processes such as nutrient uptake and ligand uptake, is used by many different viruses to gain entry into a susceptible cell and to promote infection. In earlier work it was shown that CD55 is the major attachment molecule for enterovirus 70 (EV70) on HeLa cells. EV70 is a member of the enterovirus genus. Enteroviruses use a variety of receptors and enter cells by different mechanisms. For example, poliovirus binds to the poliovirus receptor (PVR), and has been shown to enter by a novel endocytic mechanism which is clathrin-, caveolin-, and dynamin-independent but does rely on cholesterol (Danthi and Chow, 2004; DeTulleo and Kirchhausen, 1998; Kronenberger et al., 1998). Coxsackievirus A9, which binds to coxsackievirus and adenovirus receptor (CAR) and the integrin α5ß3, and coxsackievirus B4, which binds to CAR and CD55, have been shown to enter via lipid rafts (Triantafilou and Triantafilou, 2004; Triantafilou and Triantafilou, 2003); coxsackievirus B3 which also binds to CAR and CD55 has been shown to enter via clathrin-coated pits (Chung et al., 2005); and echovirus 1 which binds to the α2ß1 integrin, enters via a caveolin-mediated and/or alternative route that is dynamin- and lipid raft-dependent (Marjomaki et al., 2002; Pietiainen et al., 2004). The major objectives of the research described in this thesis were to determine if EV70 entered HeLa cells by endocytosis, and, if so, which endocytic pathway was exploited by this virus. In studies using drug inhibitors of endocytosis, chlorpromazine, which blocks clathrin-mediated endocytosis, reduced EV70 infection of HeLa cells, but cholesterol sequestering drugs had little or no effect on virus entry. These results suggested that EV70 entry was dependent on clathrin, but not on caveolae or lipid rafts. EV70 conjugated with Alexa

fluorochrome 555 appeared to co-localize with clathrin light chains tagged with enhanced yellow fluorescent protein (EYFP), consistent with a clathrin-mediated mechanism for EV70 entry into HeLa cells. Expression of the dominant-negative mutant of epidermal growth factor receptor pathway substrate clone 15 (eps15), a protein required for recruitment of adaptor protein 2 (AP-2) and epsin (both required for targeting clathrin to the cellular membrane), and assembly of clathrin-coated pits and vesicles, reduced EV70 infection of HeLa cells by 30 % when compared to the null mutant of eps15, D3Δ2. These results seem to rule in clathrin-mediated endocytosis. However, expression of the dominant-negative mutant of caveolin-1 had little or no effect on EV70 infection of cells. These results would seem to rule out caveolin-mediated endocytosis. A dominant-negative mutant of dynamin-2 strongly inhibited EV70 infection of cells, and must play an essential role in entry. Thus, it is proposed that EV70 infection may occur through an endocytic mechanism that is dependent on both dynamin and clathrin, but independent of caveolin.

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LIST OF ABBREVIATIONS

AHC	acute hemorrhagic conjunctivitis
AP180	assembly protein 180
CALM	clathrin assembly lymphoid myeloid leukemia
AP-2	adaptor protein complex 2
BPV-1	bovine papilloma virus
CAD	cationic amphiphilic drug
CTxB	cholera toxin subunit B
CAR	coxsackievirus and adenovirus receptor
CD55/DAF	decay accelerating factor
Eps15r	eps15 receptor
EGF	epidermal growth factor
EH	eps15 homology
Epsin	eps15-interacting protein
Eps15	epidermal growth factor receptor pathway substrate clone 15
ER	endoplasmic reticulum
EV70	enterovirus 70
EYFP	enhanced yellow fluorescent protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMDV	foot and mouth disease virus
GED	GTPase Effector Domain
GFP	green fluorescent protein

GPI	glycosyl-phosphatidylinositol
HIV	human immunodeficiency virus
HPEV-1	human parechovirus 1
LB	Luria-Bertani broth
LBA	Luria-Bertani broth + ampicillin
LBK	Luria-Bertani broth + kanamycin
LDL	low density lipoprotein
LLC-MK_2	rhesus monkey kidney cells
mAb	monoclonal antibody
MEM	minimal essential medium
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MOI	multiplicity of infection
MßCD	methyl-beta-cyclo-dextrin
PBS	phosphate buffered saline
PE	phycoerthryin
PFU	plaque forming unit
PH	pleckstrin homology
PRD	proline rich domain
PtdIns	phosphoinositide
PVR	poliovirus receptor
RNAi	RNA interference
Src	non-receptor tyrosine kinase identified in sarcoma caused by rous sarcoma virus

siRNA	small interfering RNA (duplexes)
SV40	simian virus 40
TBS	tris-buffered saline
Tf	transferrin
VSV	vesicular stomatitis virus

INTRODUCTION.

The earliest events in the life cycle of a virus are attachment, through receptor binding, and entry by penetration and uncoating, both of which can be important targets for antiviral therapy. Entry is an attractive target for inhibition because the entry machinery is extracellular and it is therefore easier for antiviral drugs to reach than intracellular targets. Possible entry inhibitors include proteins, peptides, carbohydrates, small organic molecules, and nucleic acid (Dimitrov, 2004).

I. Endocytosis and How Viruses Use Endocytosis.

Endocytosis, which is normally associated with cellular processes such as nutrient uptake and ligand uptake, is used by many different viruses to gain entry into a susceptible cell and to promote infection. A virus that uses endocytosis has a number of advantages: (1) it may bypass cellular defense mechanisms by camouflaging itself within a vesicle; (2) it avoids dead end cells such as erythrocytes; (3) it provides a direct route into the cellular cytoplasm and bypasses the actin cytoskeleton, cortical actin filaments and other potential cytoplasmic barriers to the perinuclear region; (4) local cues such as low pH in endosomes can help a virus undergo uncoating (Pelkmans and Helenius, 2003). Most non-enveloped viruses, like picornaviruses, then penetrate the cell by lysing the vesicular membrane, or generating a pore through which the genome can enter the cytosol (Pelkmans and Helenius, 2003). Pelkmans and Helenius have identified five different endocytic routes that viruses use to enter cells based on the involvement of the following cellular molecules or structures: clathrin, dynamin, caveolin, and lipid rafts, as shown in Table 1.

Table 1. Endocytic pathways and virus entry.

Endocytic pathways classified in accordance to requirements for clathrin, caveolin or dynamin. Examples of viruses that exploit each entry route are listed. Note that virus names in **bold** represent picornaviruses. Adapted from Pelkmans and Helenius (2003).

Endocytic Pathway	Clathrin	Caveolae	Dynamin	Lipid Rafts/ Cholesterol	Virus
Clathrin-dependent	+	-	+	-	**Human rhinovirus, parechovirus,** semliki forest virus
Caveolin-dependent	-	+	+	+	SV40, polyomavirus, **echovirus 1**
Clathrin-, caveolin-independent; Dynamin-dependent	-	-	+	+	Influenza, rotavirus, **echovirus 11**
Clathrin-, caveolin- & dynamin-independent	-	-	-	+	**Poliovirus,** SV40
Other routes (with no actin cytoskeleton involvement)	-	-	-	-	Polyomavirus, HIV, vaccinia virus

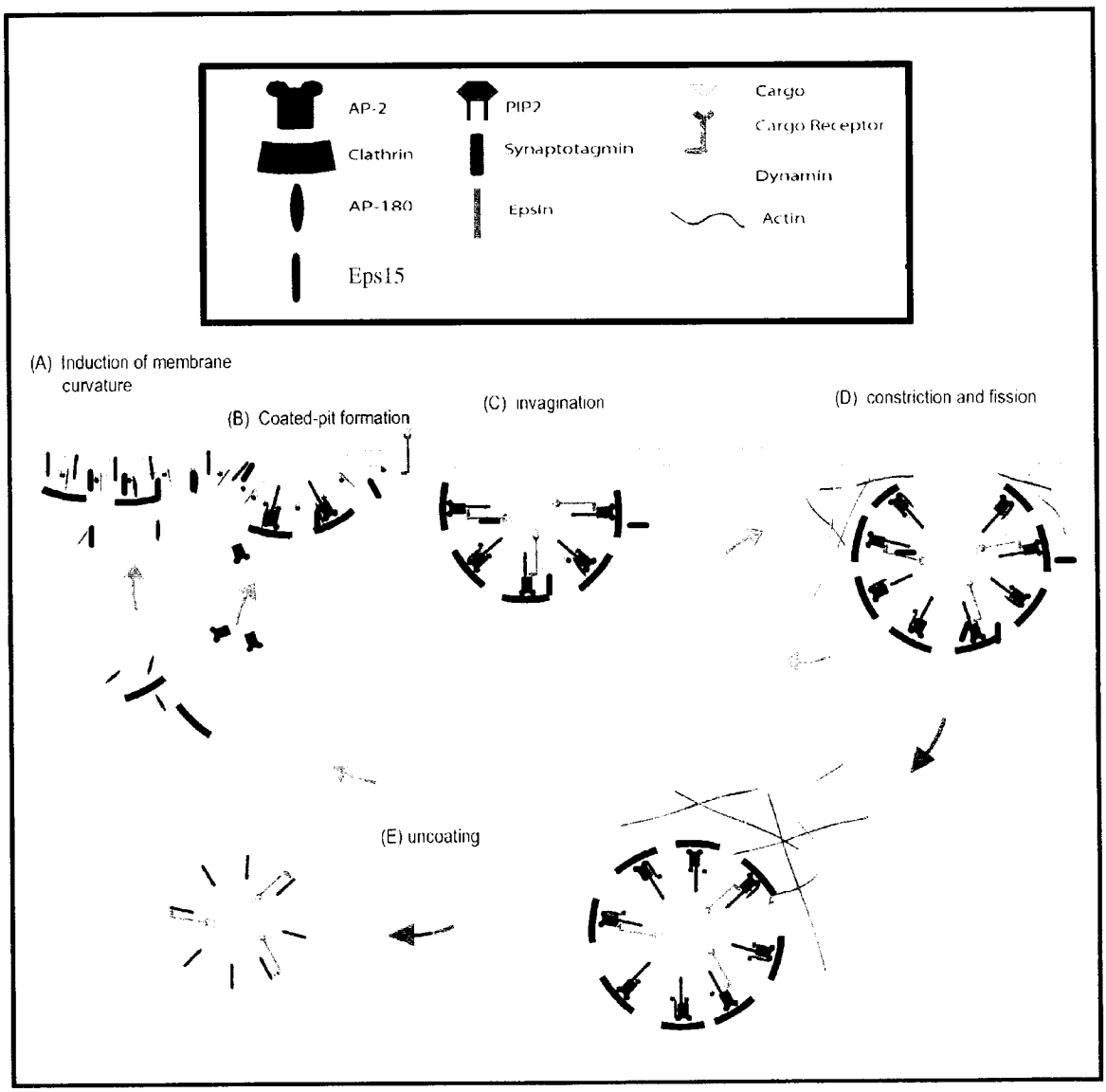
Three of these pathways are dynamin-dependent: (1) clathrin-mediated endocytosis, (2) caveolin-mediated endocytosis, (3) and a lipid raft-mediated pathway. Two dynamin-independent pathways have also been identified, one that is lipid raft-mediated and another that is independent of lipid rafts. The two most well studied endocytic mechanisms are clathrin- and caveolin-mediated endocytosis; therefore these two pathways will be discussed in some detail, keeping in mind that viruses can use any of the five routes described above.

II. Clathrin-Mediated Endocytosis.

An illustration of the assembly of clathrin-coated pits and the formation of clathrin-coated vesicles is shown in Figure 1. On average, clathrin-coated pits normally cover 0.5% to 2% of the cell surface (Brown et al., 1999). In response to an internalization signal in the cytoplasmic tail of a receptor, triggered by binding of a ligand to a receptor, clathrin is assembled on the inside face of the plasma membrane to form a characteristic invagination or clathrin-coated pit (Marsh and McMahon, 1999). There are three defined stages in clathrin-mediated endocytosis: (1) assembly of clathrin into a polygonal lattice and formation of coated pits, (2) invagination of coated pits, and (3) pinching-off of the coated vesicles. A summary of the most generally accepted model of clathrin-mediated endocytosis is as follows. A finite number of epsin molecules are first recruited to phosphoinositide (PtdIns(4,5)P_2)-rich sites at the plasma membrane following binding of a ligand to a cellular receptor, where they induce membrane budding. Simultaneously with, or subsequent to, this event, free epsin and other endocytic proteins, such as assembly protein 180/clathrin assembly lymphoid myeloid leukemia protein (AP180/CALM), are recruited to the newly formed bud. This facilitates the recruitment of clathrin-triskelions for lattice formation. The triskelions are the assembly units of

Figure 1. Model for clathrin-mediated endocytosis.

(A) Epsin induces positive membrane curvature upon binding to PtdIns(4,5)P_2-rich sites on the inner leaflet of the plasma membrane. **(B)** Epsin also recruits clathrin as well as AP-2 complexes (through direct interaction or via eps15) to the endocytic sites, where AP-2 (along with other adaptor proteins such as AP180/CALM) mediates the assembly of a clathrin cage. **(C)** The shallow coated pits invaginate in a process involving proteins such as endophilin, and dynamin. **(D)** The deeply invaginated coated pits pinch off from the plasma membrane in a dynamin-dependent manner to form clathrin-coated vesicles. Modified from Mousavi et al. (2004).


AP-2
Clathrin
AP-180
Eps15
PIP2
Synaptotagmin
Epsin
Cargo
Cargo Receptor
Dynamin
Actin
(A) Induction of membrane curvature
(B) Coated-pit formation
(C) invagination
(D) constriction and fission
(E) uncoating

clathrin; three-legged structures consisting of three heavy and three light chains. Free remaining epsin molecules in the cytoplasm then associate with adaptor protein complex 2 (AP-2) to form complexes either by direct interaction or indirectly via eps15 (epidermal growth factor receptor pathway substrate clone 15), and become displaced when AP-2 molecules trigger clathrin polymerization. At this stage the edge of the forming pit is actively engaged in the recruitment of epsin molecules, to further change the curvature of the pit, and the lattice grows in size, as a result of the recruitment of more AP-2 and clathrin, to encase the budding vesicle (Mousavi et al., 2004).

The deeply invaginated pits must be internalized by a membrane fission event, and this event is made possible by dynamin, which has GTPase activity, and two other proteins, endophilin, with acyltransferase activity, and amphiphysin, which functions as a linker between dynamin and clathrin coats (Takei et al., 1999). The model of fission is as follows: dynamin is recruited onto the forming coated vesicles through the interaction of its proline rich domain (PRD), which interacts with Src (non-receptor tyrosine kinase identified in sarcoma caused by rous sarcoma virus) homology 3 domain-containing proteins such as amphiphysin, endophilin and actin-binding proteins. Dynamin then associates with the plasma membrane via its pleckstrin homology (PH) domain. GDP/GTP exchange then causes dynamin molecules to disassociate from the coated pits and assemble at the neck of the forming coated vesicles. Upon GTP hydrolysis, the dynamin collar will constrict and cause a vesicle to pinch off from the membrane. GDP bound dynamin molecules are then disassembled (Mousavi et al., 2004). Historically, it has been assumed that clathrin-coated pit assembly is a constitutive process, but recently this view has been challenged and has been shown to perhaps also be a triggered process in some scenarios (Gonzalez-Gaitan and Stenmark, 2003; Lakadamyali et al., 2004). It

has been shown that clathrin pits can form by receptor internalization signals received through AP-2, which functions as a clathrin adaptor for the internalization of a large subset of receptors (and their ligands), for example, those with a YXXΦ motif in their cytoplasmic tails (Y=tyrosine; X=any amino acid; Φ=bulky hydrophobic amino acid) (Gonzalez-Gaitan and Stenmark, 2003; Sorkin, 2004). Ubiquitination may also serve as a signal for clathrin pits. Epsin and its associated proteins eps15 and eps15 receptor (eps15r) interact with ubiquitin, and these proteins are thus candidates for serving as clathrin adaptors for activated epidermal growth factor (EGF) receptors (Di Fiore et al., 2003). Di Fiore and colleagues studied the uptake of an EGF receptor whose cytoplasmic tail was replaced by ubiquitin. Unlike the wild type EGF receptor, this receptor was endocytosed independently of EGF binding, supporting the view that ubiquitin may function as an internalization signal for this receptor and thus it shows that clathrin routes can be a triggered process (Haglund et al., 2003).

Clathrin-coated vesicles are uncoated in the cytoplasm and deliver their cargo to the endocytic trafficking system, which consists of early endosomes, recycling endosomes, late (multivesicular) endosomes, lysosomes and the vesicles that shuttle between these compartments (Zerial and McBride, 2001; Mousavi et al., 2004).

Many viruses use clathrin-mediated endocytosis to enter cells. Typically these viruses are transported to the endosome, where a drop in pH causes the uncoating and release of the viral genome (Sieczkarski and Whittaker, 2003). Some examples of viruses that make use of the clathrin route are semliki forest virus, influenza, and bovine viral diarrhea virus (Helenius et al., 1980; Sieczkarski and Whittaker, 2002a; Grummer et al., 2004; Nunes-Correia et al., 2004). A more complete description of picornaviruses that enter cells by clathrin-mediated endocytosis will be provided subsequently (section VI).

III. Caveolin-Mediated Endocytosis.

Caveolae are non-coated, smooth, flask shaped vesicles of the plasma membrane. Caveolae are smaller than clathrin-coated vesicles, with a diameter of 50-90 nm as compared with 100-150 nm for clathrin-coated pits (Bishop, 1997). Caveolae are enriched in gangliosides, glycosphingolipids, glycosyl-phosphatidylinositol (GPI)-anchored proteins and cholesterol. The internalization cycle of caveolae is known to be regulated by kinase activity and requires an intact actin network (Rothberg et al., 1992; Parton et al., 1994).

Compared to the kinetics of clathrin-mediated entry, caveolin-mediated endocytosis is a slower process and is pH independent. Also, internalization via caveolae is not a constitutive process, unlike clathrin-mediated endocytosis, and only occurs upon cell stimulation (Thomsen et al., 2002). The major constituents of caveolae are the caveolins, phosphorylated integral membrane proteins with MW of ~21000 (Rothberg et al., 1992). There are three types of caveolin: caveolin-1 (21 kDa), which is the most common, caveolin-2 (20 kDa), which is co-expressed with and often associates with caveolin-1, and caveolin-3 (17 kDa), which is found mostly in skeletal and cardiac muscle (Scherer et al., 1996; Way and Parton, 1996).

The best studied model of this process is based on work by Pelkmans and Helenius, in 2002, on simian virus 40 (SV40). SV40 binds to major histocompatibility complex (MHC) class I molecules and moves into caveolae. The arrival of the SV40 particle in caveolae triggers phosphorylation of tyrosine residues in proteins associated with the caveolae, but the kinases and substrates involved remain to be identified. The signal transduction causes disassembly of nearby actin stress fibers. Actin is then recruited to the virus-loaded caveolae as a small actin patch, followed by bursts of actin

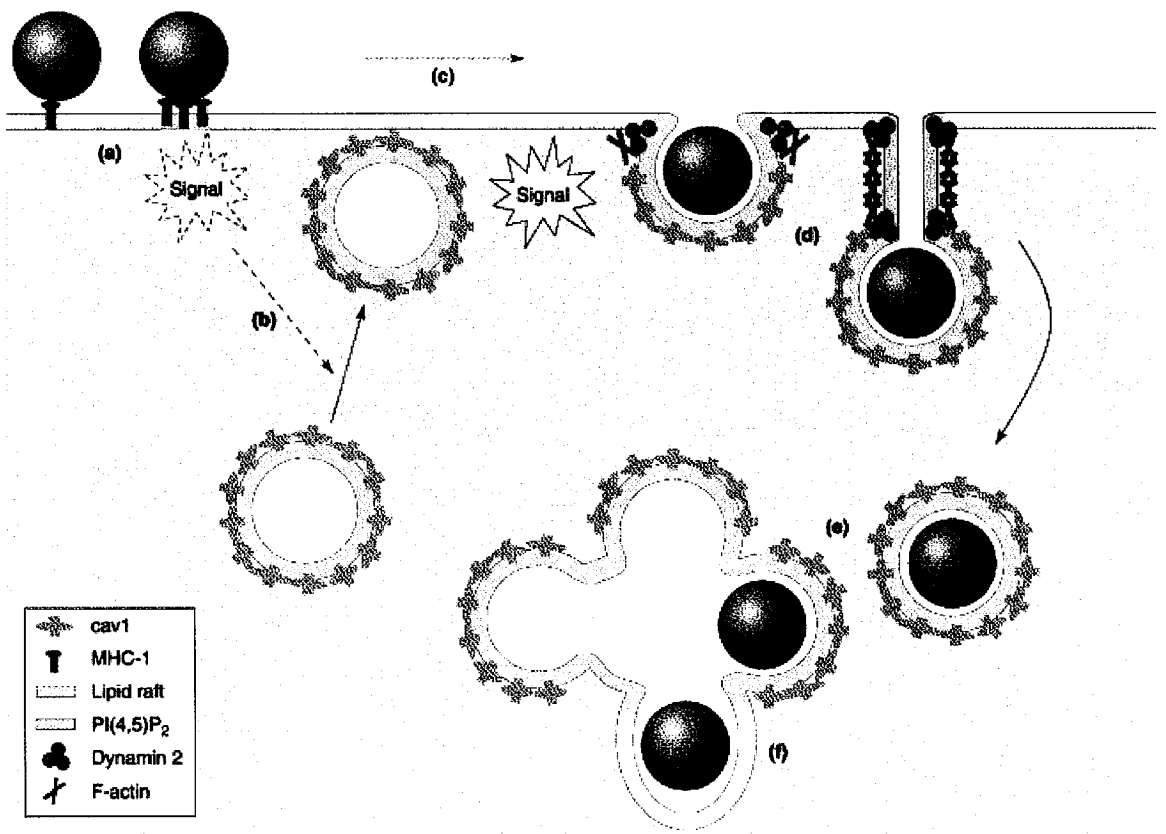
polymerization resulting in the transient appearance of actin tails emanating from the virus-loaded caveolae. Dynamin is then recruited and pinches off the caveolae, through GTP hydrolysis, to form caveolin-coated endocytic vesicles containing virus particles in the cytoplasm. The primary endocytic vesicles then transfer their viral cargo by membrane fusion, to larger, tubular membrane organelles called caveosomes (Pelkmans et al., 2001). Caveosomes are pre-existing, caveolin-1 containing organelles distributed throughout the cytoplasm. As virus accumulates in caveosomes they become increasingly dynamic. Longer tubular extensions filled with viral particles but devoid of caveolin-1 emerge and detach from the caveosomes. These vesicles are transported along microtubules to perinuclear membrane organelles identified as smooth endoplasmic reticulum (ER) (Pelkmans et al., 2001). An illustration of caveolar endocytosis is shown in Figure 2. Examples of viruses that use this route to enter host cells include SV40 (Pelkmans and Helenius, 2002), echovirus 1 (Marjomaki et al., 2002), and mouse polyomavirus (Pelkmans and Helenius, 2002).

IV. Non-Clathrin-, Non-Caveolin-Mediated Endocytosis.

Before moving on, non-clathrin-, non-caveolin-dependent routes of endocytosis deserve mention. Endocytic processes which are both clathrin- and caveolin-independent have been described. Some of these entry routes require dynamin, while others are dynamin-independent. Presently, these routes are not very well defined (Bishop, 1997; Nichols and Lippincott-Schwartz, 2001). Caveolae represent just one type of cholesterol rich microdomain on the plasma membrane. Others, more generally referred to as lipid rafts, are small stable micro-domains, 40-50 nm in diameter, and enriched in cholesterol, glycosphingolipids, sphingomyelin, GPI-linked proteins and at least some transmembrane proteins (Simons and Ikonen, 1997) that are laterally mobile and diffuse

Figure 2. Model for caveolin-mediated endocytosis.

(A) After multivalent binding to receptors, including MHC-I, SV40 (red) partitions into lipid rafts, where it might induce a signal. **(B)** Caveolin-1 positive vesicles are recruited to the membrane, into which SV40 is sequestered. **(C)** From caveolae, a tyrosine kinase signal is activated that leads to actin depolymerisation and the recruitment of an actin patch. **(D)** Local production of $PI(4,5)P_2$, the formation of a small actin tail and the recruitment of dynamin-2 lead to internalization of the caveolae. **(E)** Inside the cytosol, caveolar vesicles fuse with pre-existing caveosomes. **(F)** By an unknown process, SV40 is sorted into tubules devoid of caveolin-1, which travel to the smooth ER. Modified from Pelkmans and Helenius (2003).



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freely on the cell surface (Conner and Schmid, 2003). It has been suggested that rafts may be platforms for viral entry since they provide local concentrations of receptors and/or receptor/co-receptor complexes, as well as other cell components that can modulate entry processes (Chazal and Gerlier, 2003). Examples of viruses that use lipid rafts as platforms for cell entry are human immunodeficiency virus (HIV), SV40, echovirus 1 and rotavirus (Pelkmans et al., 2001; Marjomaki et al., 2002; Lopez and Arias, 2004; Daecke et al., 2005).

Examples of viruses employing non-clathrin-, non-caveolin-dependent endocytic pathways include influenza virus (Sieczkarski and Whittaker, 2002b; Lakadamyali et al., 2004). Other viruses that involve lipid rafts in their entry program include echoviruses types 1 and 11, rotavirus, and polyomavirus BK (Stuart et al., 2002; Marjomaki et al., 2002; Lopez and Arias, 2004; Eash et al., 2004). A summary of entry mechanisms and a list of some of the viruses that enter cells by each route are provided in Table 2.

V. Virus Entry: A Historical Perspective.

In the past number of years, new techniques for studying virus entry have been developed, resulting in a new wave of research in this area. Nevertheless, the entry mechanisms for most viruses still remain, in large part, a mystery. In this section, a synopsis of the different endocytic mechanisms available for viruses to hi-jack for entry into the cytoplasm or nucleus of a susceptible cell will be laid out. Looking at a historical perspective, a number of different techniques have been employed to analyze virus entry. The earliest types of experiments involved inhibition studies with lysomotrophic agents such as in poliovirus entry (refer to section VI). These methods led to conflicting information about the role of endocytosis and low endosomal pH in viral entry (see section VI for details)

Table 2. Examples of viruses and their entry mechanisms.

A number of different viruses and their entry mechanism, as reported in the literature. Note that virus names in **bold** represent picornaviruses.

Virus	Endocytic Pathway Used	Reference
Human rhinovirus 2 and 14	Clathrin-mediated	Snyers et al., 2003; DeTulleo and Kirchhausen., 1998
Echovirus 1	Caveolin-mediated and/or alternative route that is dynamin- and lipid raft-dependent	Marjomaki et al., 2002; Pietiainen et al., 2004
Echovirus 11	Lipid raft-mediated and/or clathrin-mediated	Stuart et al., 2002
Foot and mouth disease virus	Clathrin-mediated	O'Donnell et al., 2005
Poliovirus	Clathrin-, Caveolin-, and Dynamin-independent route	Danthi and Chow, 2004; DeTulleo and Kirchhausen, 1998; Kroenberger et al., 1998
Human parechovirus 1	Clathrin-mediated	Joki-Korpela et al., 2001
Coxsackieviruses A9 and B4	Lipid raft-mediated	Triantafilou and Triantafilou, 2003; Triantafilou and Triantafilou, 2004
Coxsackievirus B3	Clathrin-mediated	Chung et al., 2005
Major group human rhinoviruses	Dynamin-dependent but not clear whether clathrin- or caveolin- mediated	DeTulleo and Kirchhausen, 1998; Schober et al., 1998
Semliki forest virus	Clathrin-mediated	Vonderheist and Helenius, 2005.
Simian virus 40	Caveolin-mediated and/or alternative clathrin-, caveolin-, and dynamin-independent path	Pelkmans et al., 2001; Damm et al.,2005
Influenza	Clathrin-mediated and alternative caveolin-mediated path and/or clathrin-, and caveolin-independent path	Lakadamyali et al., 2004; Nunes-Correia et al., 2004; Sieczkarski and Whittaker, 2002b
Bovine viral diarrhea virus	Clathrin-mediated	Grummer et al., 2004; Lecot et al., 2005
Adenovirus 2 and 5	Clathrin-mediated	Meier and Greber, 2003

Virus	Endocytic Pathway Used	Reference
Mouse polyomavirus	Caveolin-mediated and a lipid raft- and dynamin-independent pathway	Richterova et al., 2001; Gilbert and Benjamin, 2000
Human immunodeficiency virus	Clathrin-mediated and/or direct fusion with membrane	Daecke et al., 2005
Rotavirus	Lipid raft-mediated which is clathrin- and caveolin-independent, but dynamin-dependent or direct plasma penetration	Lopez and Arias, 2004; Kwik et al., 2003
Sindbis virus	Clathrin-mediated	Snyers et al., 2003
Vesicular stomatitis virus	Clathrin-mediated	Sun et al., 2005
Vaccinia virus	Clathrin-mediated or cell-to-cell fusion	Husain and Moss, 2005
Human papillomavirus 16	Clathrin-mediated	Bousarghin et al., 2005
Porcine circovirus 2	Clathrin-mediated	Misinzo et al., 2005
Canine parvovirus	Clathrin-mediated	Parker and Parrish, 2000
West Nile virus	Clathrin-mediated	Chu and Ng, 2004
Hantaan virus	Clathrin-mediated	Jin et al., 2002
Polyomavirus BK	Caveolin-mediated that is also pH dependent	Eash et al., 2004

(Madshus et al., 1984; Perez and Carrasco, 1993; Prchla et al., 1994; Bayer et al., 1999). More recently, drugs with higher specificity for endocytic pathways have been identified. Chlorpromazine is a cationic amphiphilic drug (CAD) that causes clathrin to localize and accumulate in late endosomes, thereby inhibiting coated pit endocytosis (Wang et al., 1993; Orlandi and Fishman, 1998). Chlorpromazine has been used to show clathrin involvement in the entry of several viruses including JC virus, bovine viral diarrhea virus, human papillomavirus 16, West Nile virus, Hantaan virus and the picornaviruses, parechovirus type I and foot and mouth disease virus (FMDV) (Pho et al., 2000; Joki-Korpela et al., 2001; Jin et al., 2002; Grummer et al., 2004; Chu and Ng, 2004; Bousarghin et al., 2005; O'Donnell et al., 2005). There are several drugs that have been used to block caveolar routes. These drugs are grouped for their ability to sequester cholesterol, a prominent component of lipid rafts and caveolae, and disrupt pathways dependent on lipid rafts or caveolae. Examples include nystatin, filipin, and methyl-ß-cyclodextrin (MßCD), which actually depletes cholesterol as opposed to sequestering it (Rothberg et al., 1992; Neufeld et al., 1996; Orlandi and Fishman, 1998; Lopez and Arias, 2004). Viruses shown to enter by caveolar routes based on the use of cholesterol sequestering drugs are echovirus type I and SV40 (Anderson et al., 1996; Marjomaki et al., 2002). These drugs may have pleiotropic effects on cell function. Chlorpromazine targets many intracellular enzymes in addition to its effects on clathrin, and the cholesterol sequestering agents are likely to affect membrane functions besides endocytosis. Results of experiments that use these agents should be interpreted with some caution (Bishop, 1998; Subtil et al., 1999; Rodal et al., 1999; Sieczkarski and Whittaker, 2002a).

To overcome the potential unwanted effects of drugs in inhibiting endocytic routes and to increase specificity, a more recently developed technique has been the use of dominant-negative mutant versions of proteins that function in endocytosis. When expressed at high levels, dominant-negative mutants act by overwhelming the wild type protein, preventing its function (Sieczkarski and Whittaker, 2002a). Examples of this approach include mutant forms of dynamin- 1 and 2, K44A, which are GTPase deficient (Altschuler et al., 1998; McNiven et al., 2000). Mutant dynamins block clathrin-coated pit formation, since the GTP hydrolysis activity of dynamin is required for pinching vesicles from the plasma membrane (Schmid et al., 1998). It is now known that dynamin also serves a function in the formation of caveolae (Henley et al., 1998), the budding of golgi-derived vesicles (Jones et al., 1998; Nicoziani et al., 2000), phagocytosis (Gold et al., 1999) and non-clathrin endocytosis (Lamaze et al., 2001). Examples of virus entry pathways which are inhibited by mutant dynamin include semliki forest virus, SV40 and the picornaviruses, rhinovirus 14, and echovirus 1 (DeTulleo and Kirchhausen, 1998; Pelkmans et al., 2001; Marjomaki et al., 2002; Snyers et al., 2003). Other dominant-negative mutants, that block clathrin- or caveolin-mediated endocytosis, respectively, are eps15 and a mutant form of caveolin-1. Eps15 normally binds to the AP-2 adaptor protein which is required for internalization of clathrin-coated pits, but deleting the eps15 homology (EH) domains of eps15 produced a mutant protein that can no longer target itself to the cell membrane and thus clathrin assembly is arrested (Benmerah et al., 1999). Caveolin-dependent routes can be blocked by tagging the N terminus of caveolin-1 with green fluorescent protein (GFP), whereas fusion of GFP to the C terminus produces a protein with wild type activity (Pelkmans et al., 2001). The mechanism for this inhibitory block is unclear.

The most recent technique developed to study endocytic mechanisms has been the use of RNA interference (RNAi) to inhibit endocytosis. Robinson (Gonzalez-Gaitan and Stenmark, 2003) using RNAi knockdown of the AP-2 adaptor subunits α and μ2, found that transferrin receptor endocytosis was blocked. However, endocytosis of low density lipoprotein (LDL) and EGF receptors proceeded normally in AP-2 depleted cells (Gonzalez-Gaitan and Stenmark, 2003). Sorkin, in 2004, stated that the effect of AP-2 depletion on EGF receptor internalization was quite different depending on the internalization assay used. Measurements following short incubations of cells with labelled ligand at 37 °C revealed a partial but significant inhibition of EGF receptor internalization by AP-2 depletion. By contrast, when cells were pre-incubated with EGF at 4 °C before a 37 °C chase, AP-2 depletion was not observed to have any effect on EGF receptor internalization (Sorkin, 2004). Many coated pit proteins are difficult to down-regulate by small interfering RNA (siRNA) because they have very long half-lives and, in many cases, only partial effects on endocytosis have been observed. The absence of a siRNA effect or partial effect can also be due to functional redundancy among coated pit proteins. Several clathrin-associated proteins have close sequence homologues and multiple splicing forms. This makes the design of siRNA difficult (Sorkin, 2004).

The use of siRNA duplexes has only been used to a limited extent to examine the role of clathrin in virus entry. Entry of vesicular stomatitis virus (VSV) into cells was inhibited by siRNA duplexes directed against the clathrin heavy chain (Sun et al., 2005), and siRNAs specific for the clathrin heavy chain inhibit endocytosis of adenovirus (Meier et al., 2005) and coxsackievirus B (Yuan et al., 2005).

VI. Picornavirus Entry.

The virus that is the focus of this thesis is enterovirus 70 (EV70). EV70 is a member of the *Picornaviridae*, a family of non-enveloped viruses with single stranded RNA genomes of positive polarity, diameters of approximately 30 nm, and which contains many important human and animal pathogens. The family consists of ten genera: Apthovirus, Cardiovirus, Enterovirus, Hepatovirus, Parechovirus, and Rhinovirus plus four recently included genera, Erbovirus, Kobuvirus, Teschovirus, and Aichiviruses (Pallansch and Roos, 2001; Racaniello, 2001). The picornavirus capsid contains 60 subunits arranged in an icosahedral shape and these subunits are composed of four structural proteins, VP1, VP2, VP3, and VP4. The RNA genome of picornavirus is covalently linked at the 5' end to another protein, VPg, is polyadenylated at the 3' end, and ranges from 7200 to 8500 nucleotides in length (Racaniello, 2001).

For any group of viruses it is impossible to predict which endocytic pathway is utilized and this is clearly exemplified by the picornaviruses. Picornaviruses clearly have evolved to exploit multiple options for cell entry. They utilize a variety of different receptors, including members of the immunoglobulin superfamily, complement regulators and integrins, and many picornavirus receptors remain unidentified (Ward et al., 1994; Shafren et al., 1995; Bergelson et al., 1997; Evans, 1997; Orthopoulos et al., 2004). Not surprisingly, different picornaviruses enter cells by different endocytic pathways. Some picornaviruses enter cells via the clathrin-mediated route, some by caveolae, and others appear to utilize clathrin- and caveolin-independent entry pathways (Table 2). Of the picornavirus entry mechanisms that have been described, most involve the clathrin-mediated route. Examples of picornaviruses that enter cells via clathrin-mediated endocytosis include FMDV (O'Donnell et al., 2005), human parechovirus 1 (HPEV-1)

(Joki-Korpela et al., 2001), coxsackievirus B3 (Chung et al., 2005), and human rhinoviruses 2 and 14 (Grunert et al., 1997; DeTulleo and Kirchhausen, 1998; Snyers et al., 2003).

Echovirus 1, on the other hand, co-localizes with caveolin-1 after internalization (Bergelson J.M. et al., 1992; Marjomaki et al., 2002) and entry of coxsackievirus A21 has also been predicted to involve caveolae (Shafren, 1998).

Some picornaviruses are capable of using more than one entry route. An echovirus 11 isolate that does not use CD55 as a receptor enters cells by a clathrin-mediated endocytic route, however, CD55 binding echovirus 11 depends on lipid rafts, but not caveolin, for entry (Stuart et al., 2002). Echovirus 1, as mentioned above, uses caveolin-mediated endocytosis to enter cells; however, an alternative route that is dependent on dynamin and lipid rafts, but not caveolin, has been described (Pietiainen et al., 2004).

The endocytic pathway followed by any virus is almost certainly receptor-dependent, however, even viruses that engage the same receptor may be internalized by different mechanisms. Coxsackieviruses B3 and B4 both utilize coxsackievirus and adenovirus receptor (CAR) as an entry molecule. Coxsackievirus B3 enters cells through clathrin-coated pits (Chung et al., 2005) but coxsackievirus B4 entry is via lipid rafts and is clathrin-independent (Triantafilou and Triantafilou, 2004). How these viruses are directed to different entry pathways after binding to the same receptor remains unclear.

Probably the best-studied picornavirus is poliovirus; however, its entry mechanism is still a matter of debate and as such, poliovirus serves as an example of the difficulty in fully understanding entry of *Picornaviridae*. Early experiments made use of the electron microscope to follow poliovirus entry (Dunnebacke et al., 1969). In HEp2

and HeLa cells, virus particles were visualized inside endosome-like structures at early times post-infection, suggesting that poliovirus infection requires virus uptake into endosomes (Zeichhardt et al., 1985; Willingmann et al., 1989; Kronenberger et al., 1998). Similar results were obtained more recently with neural cells (Ohka et al., 2004). However, in these studies, it was not clear if the particles that were visualized were involved in productive or nonproductive events (Hogle, 2002). Experiments with monensin and lysomotrophic amines, which measured infectious virus, suggested that poliovirus entry required acidification (Madshus et al., 1984) but subsequent studies with bafilomycin A demonstrated that poliovirus entry occurs independent of the acidification of endosomes (Perez and Carrasco, 1993) and other studies concluded that poliovirus infection does not involve clathrin-dependent endocytosis (Curry et al., 1996; Tosteson and Chow, 1997; Belnap et al., 2000). Also, DeTulleo and Kirchhausen (1998) showed that expression of the dynamin-2 mutant, K44A, abrogated the ability of rhinovirus 14 but not poliovirus, to initiate infection, indicating that dynamin is not required for poliovirus entry (Perez and Carrasco, 1993; DeTulleo and Kirchhausen, 1998). Although poliovirus infection is thought not to be dependent on caveolae, recent studies by Danthi and Chow (2004) have shown that poliovirus infection is inhibited by MßCD and is therefore dependent on cholesterol. However, as neither the poliovirus receptor, CD155/PVR, nor viral capsid proteins were shown to localize in lipid rafts, the authors concluded that poliovirus infection was not dependent on the integrity of the rafts but rather that cholesterol may be required for transfer of viral RNA into the cytosol. Thus, it may be that poliovirus enters cells via a novel lipid raft-mediated process that is not dependent on clathrin, caveolin or dynamin.

For many picornaviruses infection is accompanied by a major structural transformation of the virion to the "altered" or "A" particle, and this change is thought to be a prerequisite for release of the viral RNA. For the enteroviruses, like poliovirus, and certain members of the major-receptor group of human rhinoviruses, this structural change is triggered by virus binding to their cognate receptors (Hoover-Litty and Greve, 1993; Powell et al., 1997; Shafren et al., 1997; Arita et al., 1999; Huang et al., 2000; Belnap et al., 2000; He et al., 2000; Xing et al., 2000; Joki-Korpela et al., 2001). For other picornaviruses, such as the minor-receptor group of human rhinoviruses, conversion to A-particles is triggered not by virus binding to its receptor but by the acidic pH within endosomes (Prchla et al., 1994; Brabec et al., 2003; Snyers et al., 2003; Hewat and Blaas, 2004; Baravalle et al., 2004). A-particle formation by other members of the major-receptor group of human rhinoviruses appears to involve aspects of both the enterovirus and minor-receptor group of human rhinovirus pathways, as A-particle formation is most efficiently achieved by a combination of virus binding to its receptor and exposure to low pH (Nurani et al., 2003). The A-particle is the predominant cell-associated form of the virus early in infection; at later times the level of A-particles begin to decrease. As A-particles decrease, a second altered form of the virus that has lost its RNA and sediments at 80S appears (Fricks and Hogle, 1990). It is generally thought that the A-particle is an intermediate in the cell entry pathway and that the 80S empty particle is the final protein product that accumulates after the RNA is released into the cytoplasm to initiate translation and replication. The idea that the A-particle is an entry intermediate is supported by the following observations: (1) A-particles bind to liposomes, providing a compelling model for membrane attachment during entry: (2) A-particles form channels in membranes, providing a mechanism to explain RNA translocation (Tosteson and

Chow, 1997; Hogle, 2002). However, the role of the A-particle as a productive intermediate in the cell entry pathway has been challenged because A-particles do not accumulate in cells when cold-adapted viruses are grown at 25 °C (Dove and Racaniello, 1997). Despite all of this information we still do not know for certain the actual site of poliovirus entry; is it from the cell surface or from an internal membrane? Nor do we know the details of the process.

VII. Enterovirus 70.

The virus that was investigated during the research described in this thesis is EV70, a causative agent of acute hemorrhagic conjunctivitis (AHC), and a member of the enterovirus genus of the family *Picornaviridae*. The enteroviruses comprise nearly seventy serotypes of human pathogens such as polioviruses, coxsackieviruses, echoviruses, and enteroviruses 68-72. They often cause mild febrile illness, but clinical manifestations of enterovirus infections also include meningitis, encephalitis, paralysis, and myocarditis (Vuorinen et al., 1999). Enteroviruses spread directly by the respiratory route or the faecal-oral route, or indirectly through contaminated hands or objects. Primary replication typically takes place in the respiratory and gastrointestinal tracts (Pallansch and Roos, 2001). On initial infection, a mild viremia occurs, usually in the alimentary tract, which can be asymptomatic. Secondary virus spread may occur through the bloodstream to target organs which leads to a major viremia and symptoms (Pallansch and Roos, 2001).

Although EV70 shares many physico-chemical characteristics with other enteroviruses, it is an atypical human enterovirus. EV70 replicates *in vitro* in a wide variety of non-primate cells such as continuous cell lines derived from rabbits, mice, hamsters, and pigs, unlike other human enteroviruses which typically have a much more

limited *in vitro* host range (Yoshii et al., 1977). EV70 may have a broad host range *in vitro*, however *in vivo* its only host is human. EV70 has a very narrow tropism, replicating primarily in the conjunctival epithelium, whereas other enteroviruses replicate in the alimentary tract and EV70 is rarely isolated from faeces unlike most other enteroviruses (Mirkovic et al., 1973). Most enteroviruses affecting humans replicate at 37 °C (Pallansch and Roos, 2001) but EV70 is the only enterovirus with a naturally occurring temperature-sensitive phenotype, replicating optimally at 33 °C, the temperature of the conjunctiva (Miyamura et al., 1974).

AHC associated with EV70 first appeared in Ghana, West Africa in 1969 and in the next three years, the disease spread explosively as a pandemic across tropical and subtropical western and central Africa, to the Middle East, and to Europe, affecting tens of millions of people. A second pandemic began in India in 1980, spread through Asia to Africa and reached the Western Hemisphere (Wright et al., 1992). In addition to these two pandemics, EV70 has been responsible for sporadic outbreaks in the past thirty two years and to date it has been implicated in roughly 100 million cases of AHC. The most recent occurrences have been reported in India, Japan and Israel (Shulman et al., 1997; Wairagkar et al., 1999; Maitreyi et al., 1999; Uchio et al., 1999).

Symptoms, which include ocular redness, epiphora (excess tearing), lid swelling, photophobia, pain, and subconjunctival hemorrhage (Wright et al., 1992), are caused by replication of the virus in the epithelial cells of the palpebral (lid) and bulbar (globe) conjunctiva and cornea. The virus destroys epithelial cells, which then erode, leading to an inflammatory response responsible for most of the clinical symptoms of AHC (Wright et al., 1992). EV70 is highly contagious and is transmitted by person to person contact through hands or objects contaminated with eye discharge (Hung and Kono, 1979).

Symptoms normally peak within twenty four hours, persist for approximately one week and resolve without complication within two weeks. Neurological symptoms develop in approximately 1/10,000 cases of AHC, complications which range from a transient, mild cranial nerve paralysis to painful lumbosacral radiculomyelitis with temporary or permanent flaccid paralysis. Although EV70 has never been isolated from cerebro-spinal fluid of patients, neutralizing antibody has been found. As well, inoculation of EV70 into the thalamus and lumbar spinal cord of cynomolgus monkeys caused motor paralysis (Kono et al., 1973) which suggests that EV70 is the cause of neurological sequelae in humans.

VIII. Hypothesis and Objectives.

Previous work in the Dimock laboratory indicates that CD55 is the major EV70 receptor on HeLa cells (Karnauchow et al., 1996; Karnauchow et al., 1998) and this was why infection was followed in this cell system. HeLa cells have been employed by a number of laboratories to elucidate viral entry mechanisms (Imelli et al., 2004; Foerg et al., 2005; Chung et al., 2005; Sun et al., 2005; Husain and Moss, 2005). The initial hypothesis was simply that EV70 would enter HeLa cells by endocytosis. Thus the major objectives of this research were:

1. To demonstrate that EV70 employs endocytosis to enter HeLa cells.
2. To characterize the endocytic pathway which EV70 uses to infect HeLa cells.

To answer the major objectives the following experimental objectives were established:

1. To develop a rapid intracellular flow cytometry protocol to detect EV70 protein synthesis as soon as possible after infection. This was desirable to minimize the potential effects of inhibitors of endocytosis on HeLa cell

functions. Flow cytometry would also be used to follow the expression of proteins involved in endocytosis and their dominant-negative mutants.

2. To develop immunofluorescent microscopy assays for EV70 and proteins involved in endocytosis. This would provide another method to confirm flow cytometry results and to follow intracellular localization of viral particles.
3. To determine if EV70 infection of HeLa cells is inhibited by drugs that block endocytosis.
4. To determine if EV70 infection of HeLa cells is inhibited by dominant-negative mutants of the following proteins involved in endocytosis: eps15, caveolin-1, and dynamin-2.
5. Because the results of drug inhibition studies pointed towards a clathrin-mediated mechanism for EV70 entry, the possibility that EV70 and clathrin co-localized early during EV70 infection was examined.

MATERIALS AND METHODS.

I. Cell Lines and Culture.

A. Sources of Cell Lines.

Human HeLa T4 cells were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program, Bethesda, MD. Rhesus monkey (*Maccaca mulatta*) kidney cells (LLC-MK_2) were originally purchased from Flow Laboratories, Rockville, MD.

B. Cell Culture.

All media and supplements were purchased from Invitrogen Life Technologies, Burlington, ON, unless otherwise stated. All cell culture manipulations were conducted in a Baker BioGard Hood. The LLC-MK_2, and HeLa T4 cell lines were cultured in growth medium that consisted of Eagle's Minimal Essential Medium (MEM) supplemented with 0.2 % (w/v) sodium bicarbonate, 2 mM L-glutamine, 50 µg/mL gentamicin sulphate and 10 % (v/v) heat-inactivated fetal bovine serum (FBS). 2X MEM consisted of growth medium with twice the concentration of the reagents described above. Cells were grown as monolayers in 100 mm Nunclon tissue culture dishes (VWR, Mississauga, ON) at 37 °C in a humidified atmosphere containing 5 % CO_2. Passaging of cells required the following: medium was aspirated and the cell monolayer was washed with pre-warmed Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose, 25 mM Tris-HCl pH 7.2). Cells were trypsinized (0.10 % trypsin and 0.53 mM EDTA in TBS, 37 °C, 3-5 minutes), removed by pipetting and transferred directly, at an appropriate dilution, to new 100 mm tissue culture plates, multi-well dishes, flasks or LabTek chamber slides (VWR). Cells were routinely split either at a 1:10 or a 1:20 dilution.

II. Virus.

A. Enterovirus 70.

The EV70 prototype strain, J670/71, was obtained from Dr. M. Hatch and Dr. M. Pallansch of the Centers for Disease Control, Atlanta, GA.

B. Virus Stocks.

LLC-MK_2 cells were grown to confluency in 175 cm^2 culture flasks (Sarstedt Inc., Montreal, QC) and infected with EV70 at a multiplicity of infection (MOI) of 0.2 for 1 hour at 32 °C in a total volume of 5 mL of growth medium per flask. The inoculum was then replaced with 20 mL of growth medium and infection was allowed to proceed for 2-3 days. Cells were then frozen at -80 °C and thawed, the sample was pelleted for 10 minutes at 4000xg at 4 °C and the virus-containing supernatant was removed. Cells were then resuspended in 5 mL of culture medium and subjected to two more freeze-thaw cycles, using a liquid nitrogen bath and a 37 °C bath, to ensure thorough lysis of the cells and maximum release of virus. Cell debris was then pelleted at 4000xg for 10 minutes at 4 °C, and this supernatant was pooled with the former. Virus stock was stored at -80 °C in aliquots of 1 mL. Virus titre was determined by plaque assay as described below.

C. Plaque Assay.

A plaque assay was used to determine infectious titres of EV70. Monolayers of LLC-MK_2 cells were grown to near confluency in Nunclon 6 well plates (VWR) at 37 °C. Growth medium was aspirated and 0.6 mL of serially diluted virus in OptiMEM was added to LLC-MK_2 cells. This was followed by an incubation at 32 °C for 1 hour after which 3 mL of 2X MEM containing 0.75 % sterile agarose (w/v) was added. Cells were then incubated for 4 days at 32 °C, and fixed for 1 hour with formol-saline (3.7 % formaldehyde, 0.8 % NaCl). Agarose was removed and cells were stained with 0.1 %

(w/v) crystal violet. Plaques were counted and virus titres were expressed in plaque forming units per mL (PFU/mL).

III. Plasmids.

A. Plasmid Constructs.

Plasmids encoding GFP-tagged caveolin-1 were kindly provided by Dr. A. Helenius (Swiss Federal Institute of Technology, Institute of Biochemistry, Zurich, Switzerland). Caveolin-1-coding sequences were inserted into pEGFP-N1 and pEGFP-C1 (Clontech, Mountain View, CA) to yield plasmids encoding caveolin-1 with wild type or dominant-negative phenotypes, respectively. Figure 3 is an illustration of the caveolin constructs. Sequences encoding GFP-tagged eps15, the dominant-negative mutants, EH29 and DIII, which inhibit clathrin-mediated endocytosis, and the null mutant, D3Δ2, in pEGFP-C2 (Clontech) were provided by Dr. A. Benmerah (Biology Unit of Cellular Interactions, Pasteur Institute, Paris, France). These constructs are illustrated in Figure 4 (Benmerah et al., 1999). Sequences encoding dynamin-2 and the dominant-negative K44A mutant, which inhibits dynamin-dependent endocytosis including both clathrin- and caveolin-mediated endocytosis, were inserted into pEGFP-N1 (Clontech). These constructs were provided by Dr. M. McNiven (Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN) and are shown in Figure 5 (Cao et al., 1998). A plasmid expressing the clathrin light chain fused to enhanced yellow fluorescent protein (EYFP) was obtained from Dr. X. Zhuang (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA). The EYFP coding sequence was fused to the N-terminus of clathrin light chain in plasmid pcDNA3.1/V5His-TOPO (Invitrogen) (Rust et al., 2004).

Figure 3. GFP-tagged caveolin-1 proteins.

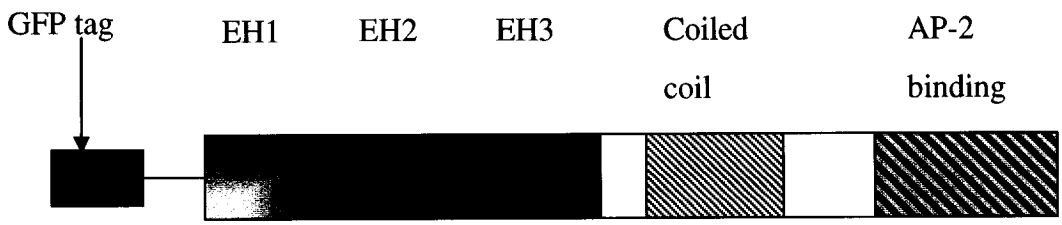
Fusion of caveolin-1 sequences to the GFP coding sequences in plasmid pEGFP-N1 **(A)** produces a C-terminal GFP-tagged caveolin with wild type activity. N-terminal fusion of GFP sequences to caveolin-1 sequences in pEGFP-C1 **(B)** produces a fusion protein with dominant-negative properties. Adapted from Pelkmans et al., 2001.

A. Wild type caveolin-1 (caveolin-1-GFP)

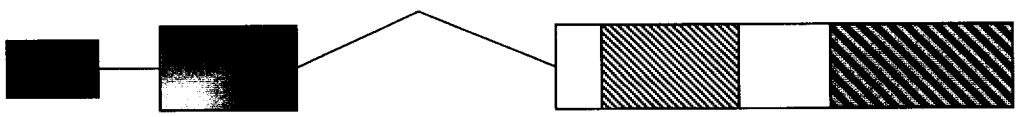
B. Mutant caveolin-1 (GFP-caveolin-1)

Figure 4. GFP-tagged eps15 proteins.

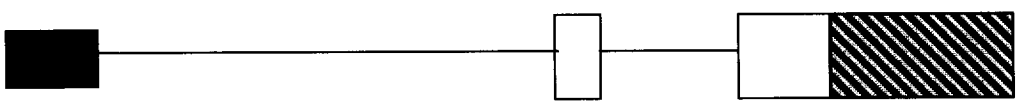
Plasmids encoding GFP-tagged wild type eps15 and mutants were constructed from pEGFP-C2. **(A)** Wild type eps15. **(B)** EH29 was constructed by removing the EH2 and EH3 domains which normally interact with NPF (Asn-Pro-Phe) motif-containing proteins including Epsin and AP180/CALM and thus mutant eps15 can no longer target itself to the cell membrane and clathrin assembly is blocked; EH29 inhibits clathrin-mediated endocytosis. **(C)** In DIII, all three EH domains as well as the coiled-coil domain are deleted. The coiled-coil domain normally allows the protein to form homo-oligomers (dimers/tetramers) and hetero-oligomers with eps15r. DIII is described as being less toxic to the cells than EH29, but was also less efficient at inhibiting clathrin-mediated endocytosis. **(D)** D3Δ2 was constructed by removing all three EH domains, the coiled-coil domain, and the AP-2 binding domain; this yields mutant eps15 that has no inhibitory activity and is used as a control. Adapted from Benmerah et al., 1999.



A. Wild type eps15



B. Mutant eps15 protein (EH29)

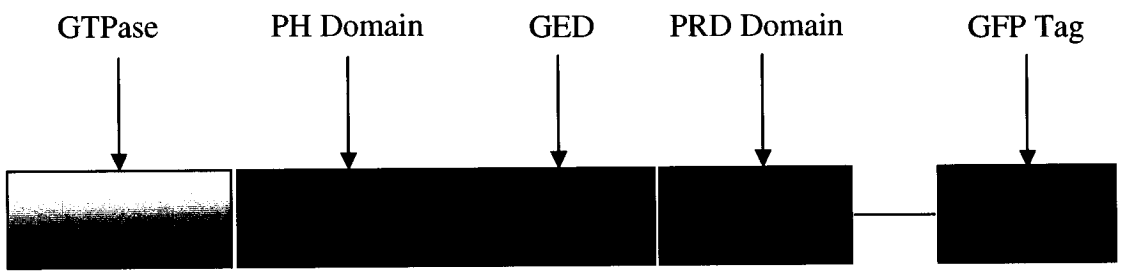


C. Mutant eps15 protein (DIII)

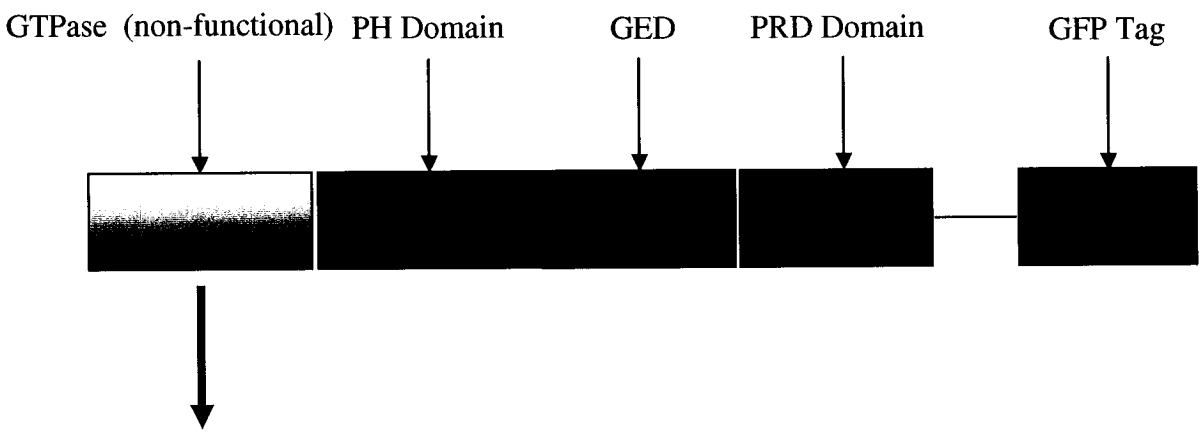
D. D3Δ2 control

Figure 5. GFP-tagged dynamin-2 proteins.

C-terminally tagged GFP dynamin-2 constructs were prepared in plasmid pEGFP-N1. (A) Wild-type dynamin-2; (B) K44A dominant-negative mutant that inhibits dynamin-dependent endocytosis, including both clathrin- and caveolin-mediated endocytosis. Adapted from Cao et al., 1998.



A. Wild type dynamin-2



B. K44A dominant-negative mutant

B. Plasmid Isolation and Purification.

Single colonies were inoculated into 3 mL of LB (Luria-Bertani broth) with 50 μg/mL kanamycin (LBK) for all plasmids except for the plasmid encoding EYFP-tagged clathrin light chain which required LB with 50 μg/mL ampicillin (LBA) in sterile 12 mL polystyrene culture tubes (VWR) and tubes were incubated with shaking at 37 °C overnight. Small scale plasmid preparations were performed on each culture as follows. Bacterial cells were pelleted at 13000 rpm for 30 seconds in a Biofuge 13 microfuge (VWR) and resuspended in 150 μL of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose, 50 μg/mL DNAse-free RNAse A). An equal volume of freshly prepared solution II (10 % SDS, 0.2 N NaOH) was added to lyse the cells by gentle mixing. The lysate was neutralized with 150 μL of solution III (5 M KAc solution, pH 4.8), mixed vigorously, and then centrifuged at 4 °C for 5 minutes at 13000 rpm. Supernatants were extracted with 400 μL of chloroform, centrifuged for 1 minute and nucleic acid was precipitated from the aqueous phase by the addition of 1 mL of ethanol. A final centrifugation of 10 minutes at 4 °C in a microfuge was performed, and pellets were resuspended in 50 μL of sterile water.

Large scale isolation of plasmid DNA was carried out using the Wizard Plus Midipreps DNA Purification System (Promega, Nepean, ON), as described by the supplier. This method is based on the binding of DNA to silica resin in the presence of salt, and elution of DNA from the resin with water (Ausubel, 1989). Plasmids were then subjected to a final ethanol precipitation, resuspended in 50 μL of sterile water and diluted as required.

IV. Flow Cytometry.

A. Flow Cytometric Analysis of EV70 Infection of HeLa Cells.

HeLa cells were dispersed by trypsinization, counted and 3 x 10^5 cells were dispensed into 5 mL polypropylene tubes (VWR). Pelleted cells were fixed and permeabilized by the addition of 500 µL of fixation/permeabilization buffer (4% paraformaldehyde, 0.1% saponin in PBS). Cells were resuspended by vortexing, incubated for 15 minutes, and pelleted. Supernatant was removed and cells were resuspended in 2 mL washing buffer (1% BSA, 0.1% NaN_3 and 0.1% saponin in PBS). Next, Gamimune solution (Bayer; provided courtesy of Dr. L. Filion, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON) was added to each tube to achieve a concentration of 100 µg/mL and cells were incubated for 5-10 minutes. Gamimune is composed of a sterilized solution obtained from pooled human blood which contains immunoglobulins and is used to reduce non-specific antibody binding and decrease background. After the incubation, 100 µL of PBS-azide (PBS containing 2% BSA, 0.1% NaN_3) containing mouse monoclonal antibody 843 (mAb843) (Chemicon, Temecula, CA; 1 µg/µl stock) diluted 1:1000 in PBS-azide was added to each tube. Cells were vortexed and washed twice with 2 mL of wash buffer. Cells were resuspended in 100 µL of PBS-azide containing a 1:1000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or a 1:500 dilution of phycoerthryin (PE)-conjugated goat anti-mouse IgG (1 µg/µL stock). Lastly, the volume was brought up to 1 mL by the addition of PBS-azide to each tube. For each cell sample, two negative controls were prepared. One negative control was the auto-fluorescence control (cells incubated in the absence of both primary and secondary antibody). The second control was the secondary antibody control

(cells incubated with secondary antibody only). Flow cytometry was performed with a Beckman Coulter EPICS XL-MCL flow cytometer with an Argon laser tuned at 15 mW of 488 nm light for excitation of fluorochromes. Usually, a minimum of 5000 events in the designated gate was collected and data were analyzed using EPICS-XL version 1.5 software (Beckman Coulter).

B. Flow Cytometric Analysis of Transfected and EV70-Infected HeLa Cells.

HeLa cells were seeded the day before transfection on a Nunclon 6 well plate (VWR) at a concentration of 3 x 10^5 cells/well. The following day, 16-24 hours later, 25 µL of OptiMEM containing 0.2 µL lipofectin/mL was incubated for 30 minutes at room temperature. Subsequently, 25 µL of OptiMEM containing plasmid DNAs (0.04 µg/µL) encoding GFP-tagged proteins or their dominant-negative mutants, were combined with the lipofectin/OptiMEM solution. After a 30 minute incubation, 650 µL of OptiMEM was added. Cells were washed with 1-2 mL OptiMEM/well and the transfection mix was added to cells. Incubation was continued at 37 °C for 3-5 hours, at which point, 3 mL of growth medium was added to each well and cells were incubated 16-24 hours at 37 °C to allow expression of GFP-tagged proteins. Cells were then infected with EV70 at a MOI of 10 for 1 hour at 32 °C. Inoculum was removed and cells were washed 3 times with 2 mL TBS, after which, 2 mL of growth medium was added, and cells were incubated at 32 °C. Six hours post infection, HeLa cells were dispersed by trypsinization, counted, dispensed into 5 mL tubes and prepared for flow cytometry as described above (section IV, A).

V. Fluorescent Microscopy.

Please note that in the following sections, all incubations with antibodies were carried out at room temperature in the dark for 30 minutes unless otherwise stated.

A. Detection of EV70 Proteins in Infected HeLa Cells.

HeLa cells were seeded, at 2 x 10^4 cells/chamber, on 8 well LabTek chamber slides (VWR). Cells were infected 2 days later at a MOI of 10-20 PFU/cell of EV70, diluted to 100 µL in OptiMEM, for 1 hour at 32 °C. Cells were then washed 3 times with 300 µL/chamber of growth medium, 300 µL/chamber of growth medium was added and cells were incubated at 32 °C for various periods of time. Cells were then fixed and permeabilized by the addition of 300 µL of cold (-20 °C) ethanol/acetone (1/1; v/v) and incubated for 2-3 minutes at 4 °C in the dark. Cells were washed twice with PBS-azide and for each well, 100 µL of a 1:200 dilution of mAb843 (Chemicon; 1 µg/µL stock) diluted in PBS-azide was added. Mouse mAb843 is an IgG with specificity for EV70 (although protein specificity has not been determined) and was used in subsequent experiments to detect viral proteins within HeLa cells. Cells were then washed with 300 µL of OptiMEM and incubated for 15 minutes with a 1:500 dilution of Alexa555-tagged goat anti-mouse IgG (1 µg/µL stock). Cells were then washed twice with 300 µL of OptiMEM. OptiMEM was aspirated and the slide was mounted using 50% glycerol/PBS containing Hoechst dye (0.1 µg/mL), to stain nuclei, and viewed. Slides were prepared for fluorescent microscopy and viewed using a Cool Snap Microscope (Olympus) or a Zeiss Axioskop 2. Photographs were taken at magnifications of 20X or 40X, with exposure times typically from 500 ms to 1 s using Image-Pro Plus v5.0 software. Commonly used filters were narrow red (UMWG2), with an excitation filter of 510-550 nm and an emission filter of 590 nm, for Alexa555; narrow green (UMNIBA3), with an

excitation filter of 470-495 nm and an emission filter of 510-550 nm, for GFP; and UV (UMWU-2), with an excitation filter of 330-385 nm and an emission filter of 420 nm, for Hoechst fluorescence.

B. Detection of EV70 Infection in Transfected HeLa Cells.

HeLa cells were seeded on 8 well LabTek chamber slides (VWR), as described above (section V, A). At approximately 40% confluency (24-48 hours later), 25 µL of OptiMEM containing plasmid DNA (4 µg/mL), encoding GFP-tagged proteins involved in endocytosis or their dominant-negative mutants, were mixed with 50 µL OptiMEM containing lipofectin or lipofectamine 2000 (both at 6 µL/mL) and incubated for 15 minutes. The mixes containing DNA and transfection reagent were combined and incubated for a further 15 minutes. Cells were washed gently with 300 µL OptiMEM and the transfection mix was added to cells. Incubation was continued at 37 °C for 3-5 hours, after which, 300 µL of growth medium was added to each well and cells were incubated 16-24 hours at 37 °C to allow for expression of GFP-tagged proteins. At 16-24 hours post transfection cells were infected with 10-20 PFU/cell of EV70 diluted to 100 µL in OptiMEM, for 1 hour at 32 °C. Cells were then washed 3 times with 300 µL of growth medium (containing no serum or gentamicin sulphate), 300 µL of growth medium was added and cells were incubated at 32 °C. Between 6-12 hours post-infection, cells were fixed, permeabilized and stained for EV70 proteins with mAb843 as described above (section V, A). Slides were prepared for microscopy and photographed as described above (section V, A).

C. Co-Localization of Alexa555-Labelled EV70 and EYFP-Tagged Clathrin Light Chain.

HeLa cells growing on LabTek chamber slides (VWR) were washed with 300 µL OptiMEM and 5 µL/chamber of Alexa555-labelled EV70 diluted to 75 µL with OptiMEM (~0.05 µg/µL stock) was added. Alexa555-labelled EV70 was received as a gift from Dr. Reza Nokhbeh (Department of Biochemistry, Microbiology and Immunology, University of Ottawa). Adsorption of virus was carried out in the dark at 4 °C for 1 hour, followed by 3 washes with 300 µL of OptiMEM, after which 300 µL of growth medium was added and incubation at 32 °C was carried out for different periods of time. The cells were then washed with 300 µL of OptiMEM twice and fixed in 3.7% paraformaldehyde/PBS at room temperature in the dark for 15 minutes. The fixing solution was aspirated and slides were prepared for microscopy.

HeLa cells were transfected with plasmid expressing an EYFP-clathrin light chain fusion protein, as described above (section V, B). HeLa cells expressing EYFP-clathrin light chain were then infected with Alexa555-labelled EV70 and at various time points post-infection, cells were washed with 300 µL of OptiMEM twice and fixed in 3.7% paraformaldehyde/PBS at room temperature in the dark for 15 minutes. The fixing solution was aspirated and slides were prepared for microscopy (section V, A).

VI. Treatment of HeLa Cells with Endocytosis Inhibitors.

Growth medium (with no FBS) containing the appropriate drug (refer to Table 3) was added to HeLa cells in 6 well plates in a final volume of 500 µL. Cells were incubated for 30 minutes at 37 °C and then infected with EV70 at a MOI of 10, with drug present, in a volume of 200 µL, for 1 hour at 32 °C. Inoculum was removed and cells

were washed 3 times with 2 mL TBS, after which, 2 mL of growth medium, with drug present, was added and cells were incubated at 32 °C. Six hours post infection, cells were fixed with 3.7 % paraformaldehyde and prepared for flow cytometry as described above (section IV, A). In experiments carried out in 8 well chamber slides for fluorescent microscopy, all volumes were scaled down to 100 µL for washes and inocula, and 300 µL for incubations. Slides were prepared for microscopy as described above (section V, A).

Table 3. Drug inhibitors of endocytosis.

Drug	Endocytic Route Inhibited
Chlorpromazine	Clathrin
Filipin	Caveolae
Nystatin	Caveolae
MßCD	Caveolae

Cell viability in the presence of drug inhibitors was determined by trypan blue assay. HeLa cells were trypsinized and mixed by pipetting up and down and then 10 µL of cells were mixed with 10 µL of trypan blue (Sigma, Oakville, ON) solution (0.1 % (w/v) trypan blue in PBS). Cells were counted in a hemacytometer; blue cells were considered non-viable and colourless cells were considered viable.

VII. Markers for Clathrin- and Caveolin-Mediated Endocytosis.

A. Transferrin Uptake by HeLa Cells.

Uptake of transferrin (Tf) was used as a marker for clathrin-mediated endocytosis (Benmerah et al., 1999; Roy et al., 1999; Pelkmans et al., 2001; Joki-Korpela et al., 2001; Triantafilou and Triantafilou, 2003; Singh et al., 2003; Snyers et al., 2003; Berryman et al., 2005). For fluorescent microscopy, growth medium was aspirated from HeLa cells

and cells were washed with 300 µL of TBS. Then, 300 µL of serum free growth medium was added and cells were incubated at 37 °C for 30 minutes to deplete endogenous Tf. Cells were incubated with 0.5 µg/mL of Alexa555-labelled Tf in a final volume of 100 µL of serum free growth medium for 15 minutes at 37 °C. Cells were washed twice with 300 µL of acidic buffer (0.1 M Glycine, 0.1 M NaCl, pH 3.0) to remove uninternalized Tf. Cells were fixed with 3.7% paraformaldehyde in 50% glycerol/PBS, washed with PBS-azide and prepared for microscopy as described above (section V, A).

For flow cytometry, volumes were scaled up to 2 mL for washes and 500 µL to 1 mL for incubations. HeLa cells growing in 6 well plates were washed and replaced with serum free growth medium to deplete endogenous Tf prior to incubation with 50 µg/mL biotinylated Tf. Uninternalized Tf was removed, and cells were fixed, permeabilized, and trypsinized. Cells were then incubated with a 1:500 dilution of PE-conjugated strepavidin (1 µg/µL stock) and analyzed by flow cytometry as described above (section IV, A).

B. Cholera Toxin Subunit B Uptake by HeLa Cells.

Uptake of cholera toxin subunit B (CTxB) was used as a marker for caveolin-mediated endocytosis (Orlandi and Fishman, 1998; Fittipaldi et al., 2003; Singh et al., 2003; Pietiainen et al., 2004; Berryman et al., 2005; O'Donnell et al., 2005). Uptake of Alexa555-tagged CTxB (final concentration 0.2 µg/mL) was monitored by fluorescent microscopy as described above (sections V, A and VII, A). A suitable flow cytometry procedure was not developed for CTxB uptake.

Note that a student t-test was performed where it was appropriate to calculate P values.

RESULTS.

I. Flow Cytometry Analysis for Monitoring EV70 Entry into HeLa cells.

The overall objectives of the research presented in this thesis were to demonstrate that EV70 employs endocytosis to enter HeLa cells and to characterize the endocytic pathway used by EV70. Previously, the EV70 receptor on HeLa cells was shown to be CD55 (Karnauchow et al., 1996). Because at least one other picornavirus that binds to CD55, coxsackievirus B3, has been shown to enter HeLa cells by endocytosis (Chung et al., 2005), it was hypothesized that EV70 would also gain access to the interior of a host cell by an endocytic process, rather than by direct delivery of viral RNA through the plasma membrane, as has been proposed for poliovirus (Hogle, 2002; Bubeck et al., 2005; Tuthill et al., 2006).

The first experimental objective was to establish a protocol for following EV70 entry into HeLa cells. It was desirable to have an assay that would detect EV70 infection within hours of exposing cells to virus, and that could replace traditional plaque assays. Since plaque assays take four days for plaques to develop, it was thought that differences in virus entry might be minimized by long incubation times. Long exposure to inhibitors during plaque assays might also have detrimental effects on cell functions other than endocytosis and on viability. Intracellular flow cytometry appeared to be a logical choice for the detection of newly synthesized EV70 proteins in HeLa cells after infection; flow cytometry is sensitive and quantitative, and a monoclonal antibody (mAb843) specific for EV70 was available. To follow EV70 infection, HeLa cells in 6 well plates were infected with EV70, and at various time points after infection, cells were harvested, fixed, permeabilized and stained for EV70 protein synthesis. Analysis was carried out by flow cytometry as shown in Figures 6 and 7.

Figure 6. EV70 protein synthesis monitored by intracellular flow cytometry.

HeLa cells growing on 6 well plates were exposed to EV70 (MOI=10) for 1 hour at 32 °C following a 30 minute incubation in the presence or absence of 20 μg/mL cycloheximide. Cells were washed to remove non-adsorbed virus, and fresh growth medium, with or without cycloheximide, was added. Six hours after infection, cells were detached with trypsin-EDTA, fixed and permeabilized. EV70 protein synthesis was monitored by intracellular flow cytometry using mAb843 and PE-conjugated goat anti-mouse IgG. Uninfected and auto-fluorescence controls were negative, but are not shown, for simplicity. Control represents cells stained with secondary antibody only and treated with cycloheximide. Untreated represents cells infected with EV70. Experiment was performed once.

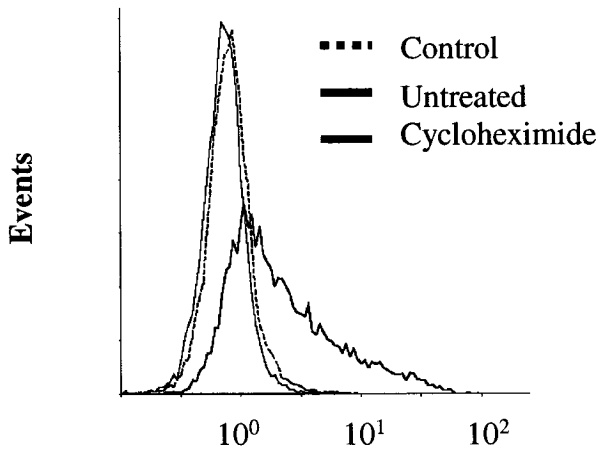
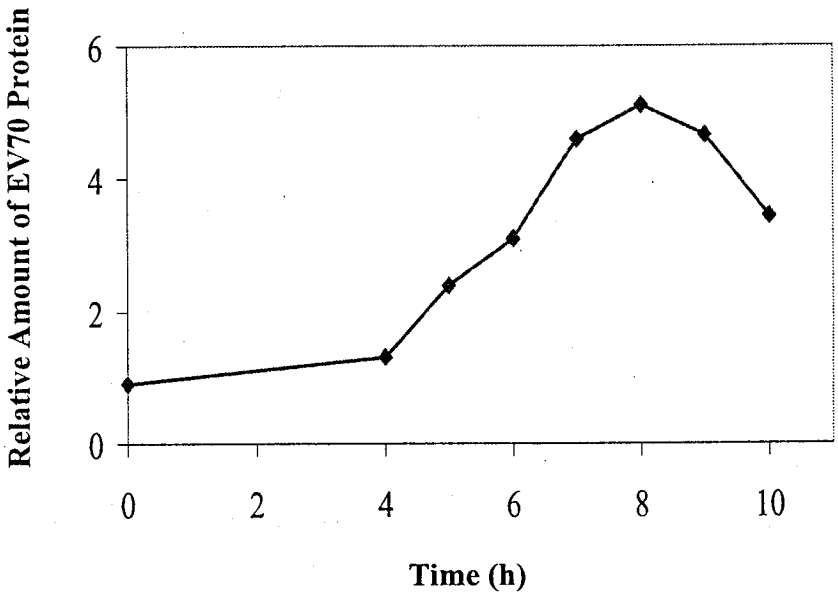

Control
Untreated
Cycloheximide
Events
10^0
10^1
10^2
Mean Fluorescence Intensity

Figure 7. Time course of EV70 infection monitored by intracellular flow cytometry.

HeLa cells growing on 6 well plates were infected with EV70 (MOI=10) at 32 °C for one hour. Cells were washed to remove non-adsorbed virus, and fresh growth medium was added. At various time points, cells were detached with trypsin-EDTA, fixed and permeabilized. EV70 protein synthesis was monitored by intracellular flow cytometry using mAb843 and PE-conjugated goat anti-mouse IgG. Experiment performed once. Values for the relative amount of EV70 protein detected in cells at each time point were calculated as follows:

$$\frac{\text{(mean fluorescence intensity (MFI) with } 1^{\circ}\text{ Ab} + 2^{\circ}\text{ Ab})/(\text{MFI with } 2^{\circ}\text{ Ab only) at time } x}{\text{(mean fluorescence intensity (MFI) with } 1^{\circ}\text{ Ab} + 2^{\circ}\text{ Ab})/(\text{MFI with } 2^{\circ}\text{ Ab only) at time 0}}$$


Relative Amount of EV70 Protein
6
4
2
0
0
2
4
6
8
10
Time (h)

Subsequently, six hours post infection was chosen for routine analysis since high fluorescence above background levels was consistently observed at this time point, after infection of HeLa cells at a MOI of 10. As well, viral protein synthesis begins to plateau between 7-8 hours after infection, thus the six hour time point would be the most sensitive section of the slope for analysis. To confirm that synthesized viral protein, and not input virus, was being detected, cycloheximide was used to inhibit protein synthesis. As shown in Figure 6, cycloheximide strongly inhibited expression of EV70 proteins in infected cells demonstrating that mAb843 is detecting viral proteins synthesized following infection. A second EV70-specific antibody, mAb3A7 (Karnauchow et al., 1996), gave similar results (data not shown) but with a higher background. This background was due to nuclear staining in uninfected cells (personal communication, Dr. Reza Nokhbeh). Antibody mAb843 was used in all subsequent experiments.

II. Inhibition of EV70 Infection by Chlorpromazine.

As a first step to characterizing the entry process of EV70, chlorpromazine, which has been reported to inhibit clathrin-mediated endocytosis (Wang et al., 1993) and has been used by others to demonstrate clathrin-mediated virus entry (Pho et al., 2000; Joki-Korpela et al., 2001; Sieczkarski and Whittaker, 2002; Triantafilou and Triantafilou, 2004; Sanchez-San Martin et al., 2004), was tested for its ability to interfere with EV70 infection. The synthesis of EV70 proteins in HeLa cells would indicate that EV70 had entered cells and initiated the infectious cycle.

HeLa cells were incubated in the presence or absence of chlorpromazine for 30 minutes prior to infection with EV70. Six hours after infection, cells were harvested and EV70 proteins were detected by intracellular flow cytometry, as described above (section

I). Uptake of biotinylated Tf, a classic marker of clathrin-mediated endocytosis (Ghosh et al., 1994; Johannes and Lamaze, 2002), was also examined by intracellular flow cytometry using PE-conjugated strepavidin, 15 minutes after exposure of the cells to Tf. As shown in Figure 8, chlorpromazine reduced both the expression of EV70 proteins and Tf uptake in HeLa cells by >50%, as compared to untreated HeLa cells. These results are consistent with the idea that EV70 uses a clathrin-mediated route as a major mechanism to enter HeLa cells.

The effects of chlorpromazine on EV70 entry into HeLa cells were confirmed by immunofluorescent microscopy. EV70 protein synthesis and Tf uptake were both greatly reduced in chlorpromazine-treated cells, as compared to untreated cells (Figure 9). To determine if the effects of chlorpromazine on EV70 infection of HeLa cells resulted in reduced virus production, chlorpromazine-treated and untreated HeLa cells were infected with EV70. Twenty hours after infection, in the presence or absence of chlorpromazine, cell lysates were prepared by freezing and thawing, and virus titres were determined by plaque assay. As shown in Figure 10, chlorpromazine treatment inhibited EV70 production by over 60%, supporting the interpretation that EV70 enters HeLa cells by clathrin-mediated endocytosis.

The possibility existed that chlorpromazine was toxic to HeLa cells under the experimental conditions used. Therefore, viability of HeLa cells during incubation in the presence of chlorpromazine was assessed by trypan blue staining. As shown in Figure 11, HeLa cells retained their viability for at least 6 hours in the presence of chlorpromazine at concentrations up to and exceeding 50 μM; however, viability decreased significantly with longer exposures to 50 μM chlorpromazine. This suggests that the effects of prolonged exposure to chlorpromazine on cell viability, rather than direct effects on EV70

Figure 8. Chlorpromazine inhibits transferrin uptake and EV70 entry: flow cytometry.

A. *Flow cytometric analysis of Tf uptake.* HeLa cells growing on 6 well plates were treated with biotinylated Tf (50 μg/mL) following a 30 minute incubation in the presence or absence of 50 μM chlorpromazine at 32 °C. After 15 minutes in the continued presence of drug, cells were washed with acidic buffer to remove external Tf, fixed and permeabilized. Biotinylated Tf was detected with PE-conjugated strepavidin.

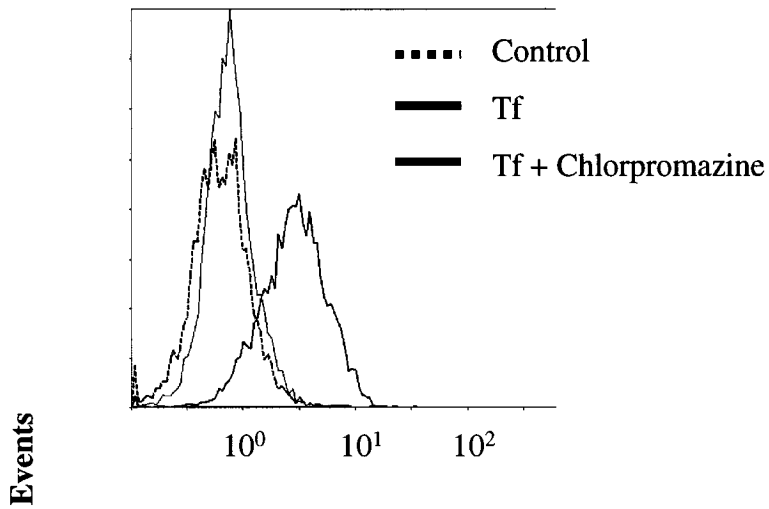
B. *Flow cytometric analysis of EV70 entry.* HeLa cells growing on 6 well plates were infected with EV70 (MOI=10) following a 30 minute incubation in the presence or absence of 50 μM chlorpromazine at 32 °C. After an additional 6 hours, in the continued presence or absence of chlorpromazine, cells were fixed and permeabilized. EV70 protein synthesis was monitored by intracellular flow cytometry using mAb843 and PE-conjugated goat anti-mouse IgG.

C. *Inhibition of Tf and EV70 uptake by chlorpromazine.* Uptake of Tf or EV70 was calculated as follows:

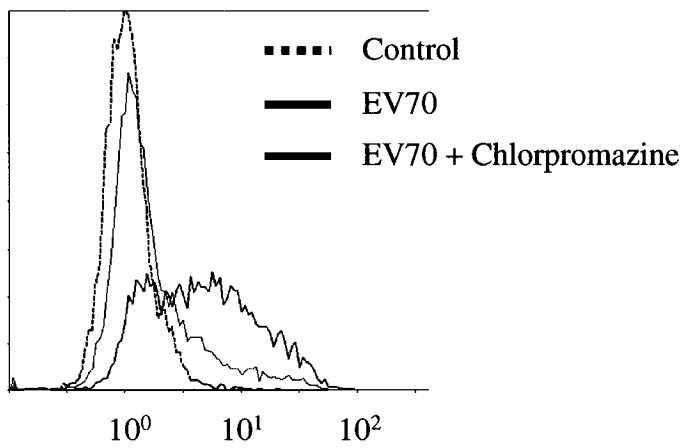
$$\frac{\text{MFI treated cells (1°Ab + 2°Ab)/MFI treated cells (2°Ab only)}}{\text{MFI untreated cells (1°Ab + 2°Ab)/MFI untreated cells (2°Ab only)}} \times 100$$

The results are presented as mean percentages ± standard deviation for four independent experiments. Control represents cells stained with secondary antibody only. P value < 0.05

A.



B.



Mean Fluorescence Intensity

C.

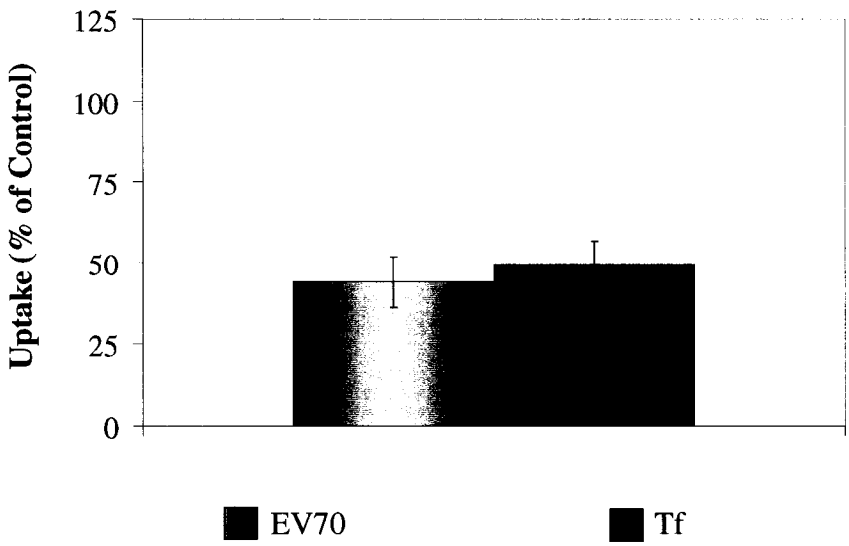


Figure 9. Chlorpromazine inhibits transferrin uptake and EV70 entry: fluorescent microscopy.

A. HeLa cells growing on LabTek chamber slides were incubated with Alexa555-labelled Tf (0.5 μg/mL, red) following a 30 minute incubation in the presence or absence of chlorpromazine at 32 °C. After an additional 15 minutes, in the presence or absence of chlorpromazine, cells were washed with acidic buffer to remove external Tf, fixed and permeabilized, and visualized by fluorescent microscopy at 40X magnification. Note that images are zoomed in four fold. Nuclei were stained with Hoechst dye (0.1 μg/mL, blue).

B. HeLa cells growing on LabTek chamber slides were infected with EV70 (MOI=10) following a 30 minute incubation in the presence or absence of chlorpromazine at 32 °C. After an additional 6.5 hours in the presence or absence of chlorpromazine, cells were fixed and permeabilized. EV70 proteins were visualized by fluorescent microscopy at 40X magnification after staining with mAB843 and Alexa555-conjugated goat anti-mouse IgG (red). Note that images are zoomed in four fold. Nuclei were stained with Hoechst dye (0.1 μg/mL, blue).

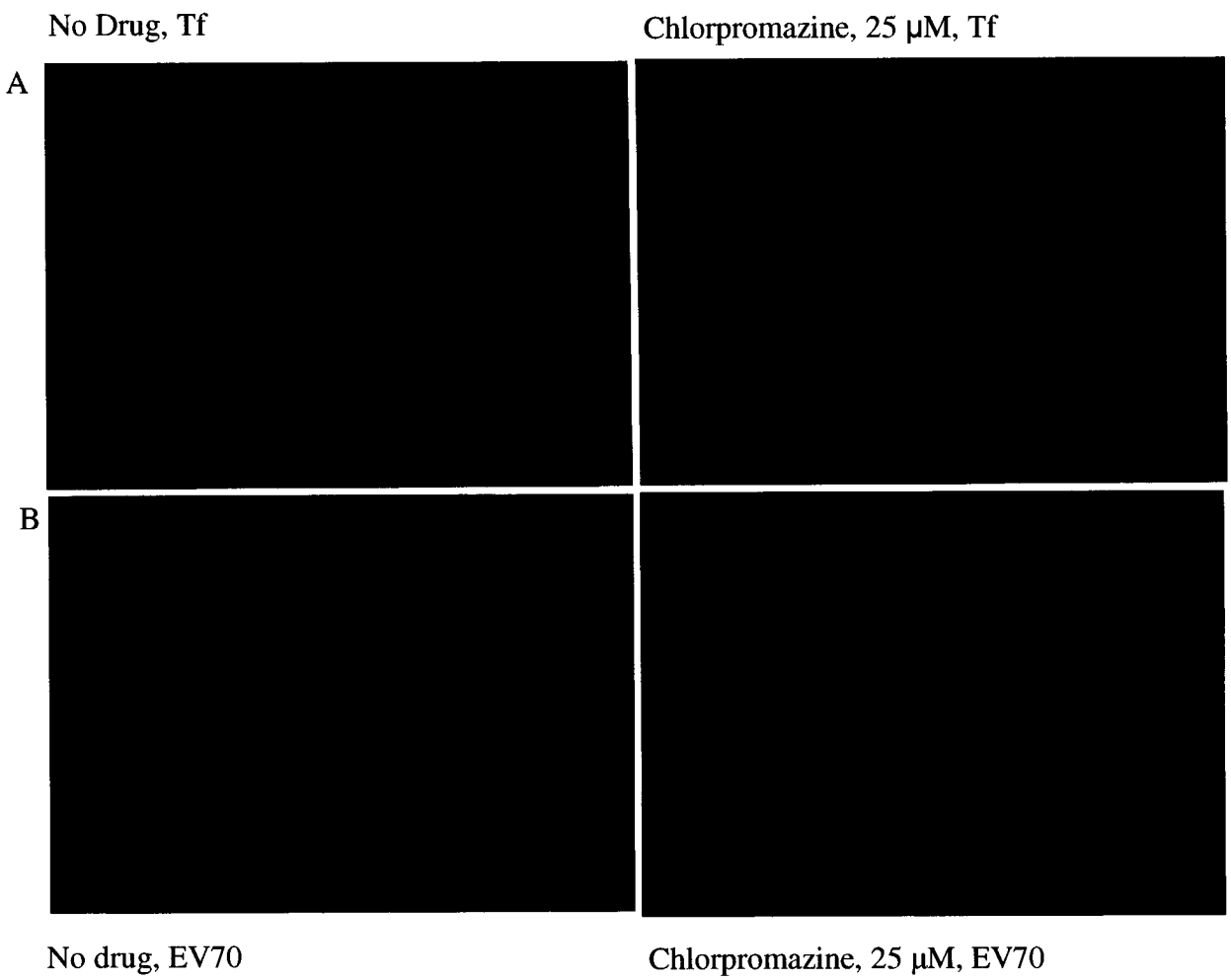

No Drug, Tf
Chlorpromazine, 25 μM, Tf
A
B
No drug, EV70
Chlorpromazine, 25 μM, EV70

Figure 10. The effects of endocytosis inhibitors on EV70 infection of HeLa cells.

HeLa cells growing on 6 well plates were treated with inhibitors of endocytosis, as indicated, for 45 minutes at 37 °C. Cells were then transferred to 32 °C and infected with EV70 at a MOI of 5. Cells were incubated, in the presence or absence of drug for 20 hours, and then frozen and thawed 3 times to release virus. Virus titres were determined by plaque assay on LLC-MK_2 cells. Filipin and nystatin are cholesterol sequestering drugs that inhibit caveolin-mediated endocytosis. Experiment performed once. Virus yield (% of control) was calculated as follows:

[(PFU of treated cells)/(PFU of untreated cells)] x 100

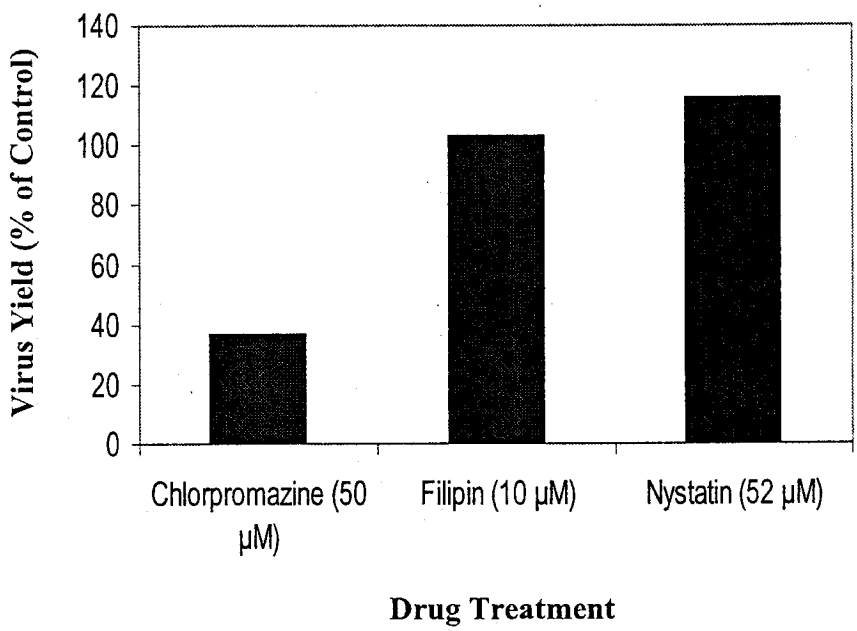

Virus Yield (% of Control)
140
120
100
80
60
40
20
0
Chlorpromazine (50 µM)
Filipin (10 µM)
Nystatin (52 µM)
Drug Treatment

Figure 11. Effects of endocytosis inhibitors on EV70 entry and cell viability.

A-C: HeLa cells growing on 6 well plates were incubated for 30 minutes at 32 °C, in the presence or absence of inhibitors of endocytosis, at different concentrations. Some cells were infected with EV70 (MOI=10). After an additional six hours, in the presence or absence of drug, cells were fixed and permeabilized. EV70 protein synthesis, as an indicator of EV70 entry, was monitored by intracellular flow cytometry using mAb843 and PE-conjugated goat anti-mouse IgG, and was calculated as follows:

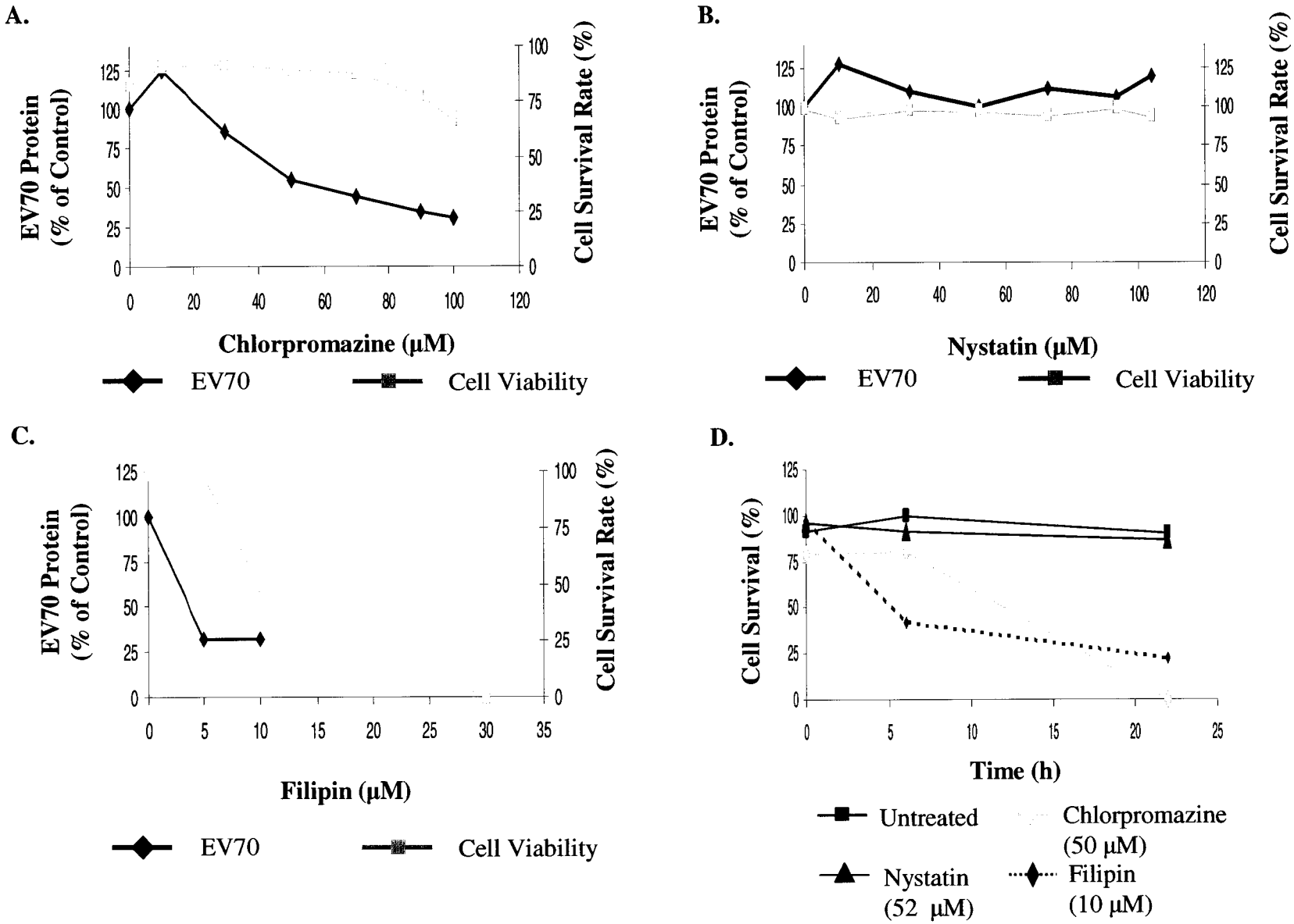
$$\frac{(\text{MFI treated cells } (1^{\circ}\text{Ab} + 2^{\circ}\text{Ab}))/(\text{MFI treated cells } (2^{\circ}\text{ Ab only})}{(\text{MFI untreated cells } (1^{\circ}\text{Ab} + 2^{\circ}\text{Ab}))/(\text{MFI untreated cells } (2^{\circ}\text{Ab only})} \times 100$$

The uninfected HeLa cells were detached from plates with trypsin-EDTA and cell viability was determined by trypan blue assay. Cell Survival (%) = [number of live cells/(number of live cells + dead cells)] x 100.

A. Chlorpromazine; **B**. Nystatin; **C**. Filipin.

D. HeLa cells were incubated with endocytosis inhibitors, at the indicated concentration. At various times during incubation cells were detached with trypsin-EDTA and cell viability was assessed by trypan blue assay.

These experiments were performed once.


A.
EV70 Protein (% of Control)
Cell Survival Rate (%)
Chlorpromazine (μM)
EV70
Cell Viability
B.
EV70 Protein (% of Control)
Cell Survival Rate (%)
Nystatin (μM)
EV70
Cell Viability
C.
EV70 Protein (% of Control)
Cell Survival Rate (%)
Filipin (μM)
EV70
Cell Viability
D.
Cell Survival (%)
Time (h)
Untreated
Chlorpromazine (50 μM)
Nystatin (52 μM)
Filipin (10 μM)

entry, account for the inhibition of EV70 production that was observed. Thus, this reinforces the idea that flow cytometry and immunofluorescent microscopy are more reliable methods for evaluating the impact of endocytosis inhibitors on virus entry.

III. Cholesterol Sequestering Drugs Have Little or No Effect on EV70 Infection into HeLa Cells.

The possibility that EV70 may use caveolin- or lipid raft-mediated endocytosis for entry was also examined. Three different cholesterol sequestering reagents (nystatin, filipin and MβCD; Table 3) were tested for their ability to block EV70 infection of HeLa cells. The B subunit of cholera toxin (CTxB) is commonly used as a marker for caveolar endocytosis (Hinshaw and Schmid, 1995; Schnitzer et al., 1996; Henley et al., 1998; Orlandi and Fishman, 1998; Pang et al., 2004; Sanchez-San Martin et al., 2004; Berryman et al., 2005). Therefore the effects of the cholesterol sequestering drugs on CTxB uptake were also examined. Cells were incubated in the presence or absence of the different drugs and either infected with EV70 or exposed to CTxB. Entry of EV70 was assayed by intracellular flow cytometry, immunofluorescent microscopy and plaque assay. Uptake of Alexa555-conjugated CTxB was monitored by fluorescent microscopy. All three drugs appeared to reduce the amount of fluorescent CTxB taken up by HeLa cells, confirming the effectiveness of the conditions of treatment (Figure 12). Neither nystatin nor filipin had any noticeable effect on EV70 protein synthesis as assayed by immunofluorescent microscopy (Figure 12), and this was confirmed for nystatin by flow cytometric analysis (Figure 13). As well, filipin and nystatin treatment of HeLa cells had no noticeable effects on the yield of EV70 (Figure 10). Both immunofluorescent microscopy (Figure 12) and flow cytometry (Figure 13) showed that EV70 entry into

Figure 12. Effects of cholesterol sequestering agents on CTxB uptake and EV70 entry.

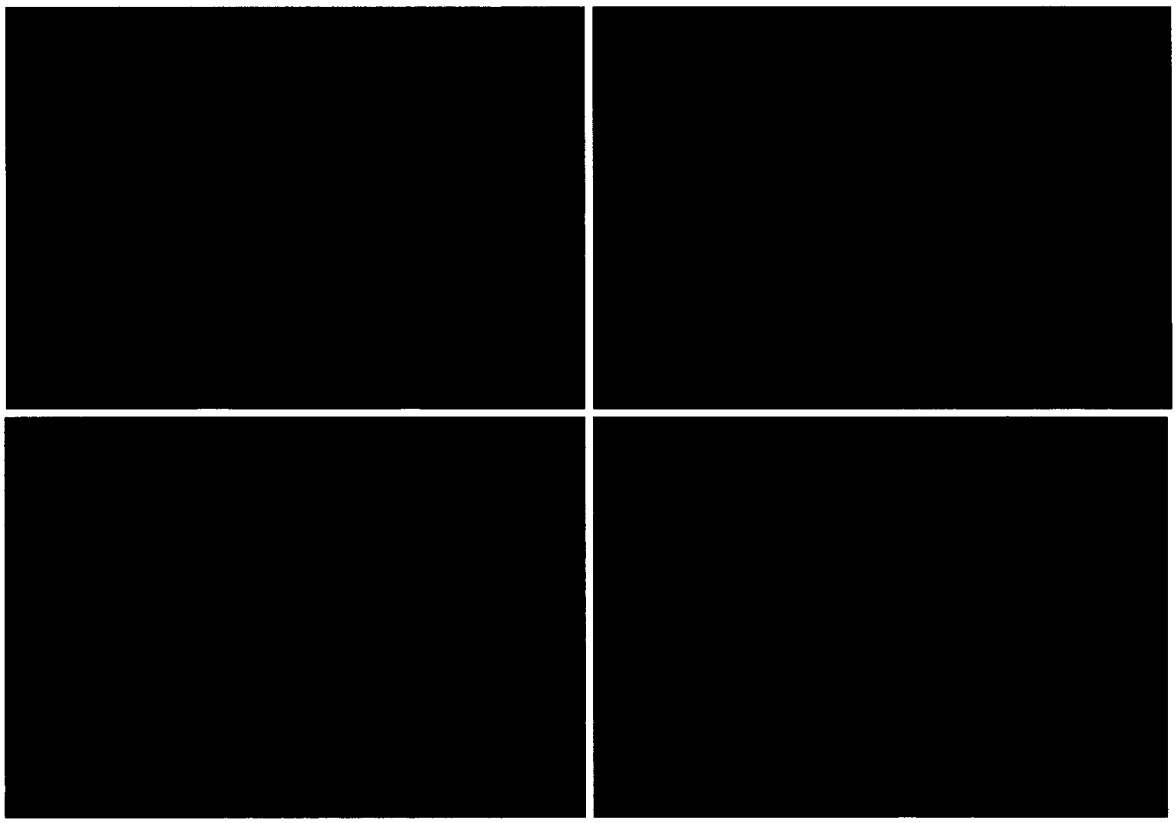
A. HeLa cells growing on LabTek chamber slides were incubated with Alexa555-conjugated CTxB (0.2 μg/mL, red) following a 30 minute incubation in the presence or absence of each drug, at the indicated concentrations. After an additional 15 minutes, in the continued presence of drug, cells were washed with acidic buffer to remove external CTxB, fixed and permeabilized, and CTxB uptake was visualized by fluorescent microscopy at 40X magnification.

B. HeLa cells growing on LabTek chamber slides were infected with EV70 (MOI=10) following a 30 minute incubation in the presence or absence of each drug, at the indicated concentrations, at 32 °C. Six and a half hours after infection, in the continued presence of drug, cells were fixed and permeabilized. EV70 proteins were visualized by fluorescent microscopy at 40X magnification after staining with mAb843 and Alexa555-conjugated goat anti-mouse IgG (red). Nuclei were stained with Hoechst dye (0.1 μg/mL, blue).

A.

a. Untreated, DMSO cntl, CTxB

b. MßCD, 5 mM, CTxB



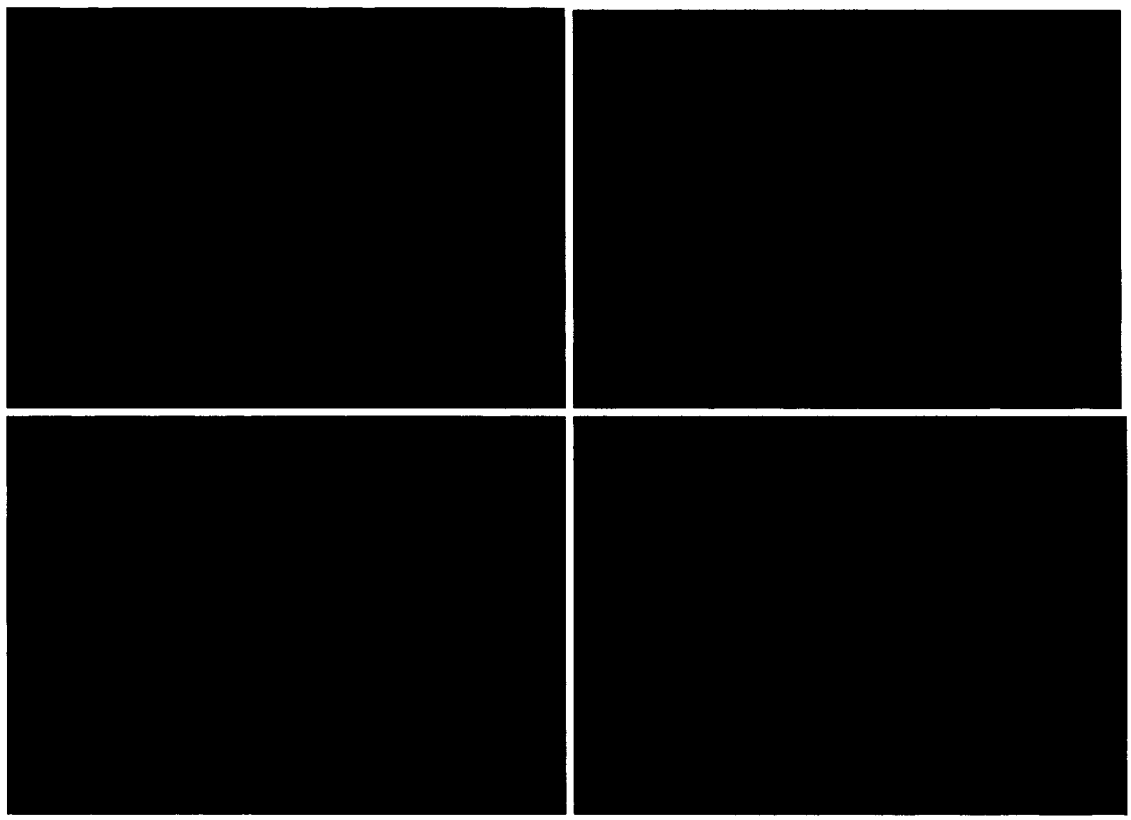
c. Filipin, 10 μM, CTxB

d. Nystatin, 104 uM, CTxB

B.

a. Untreated, DMSO cntl, EV70

b. MßCD, 5 mM, EV70



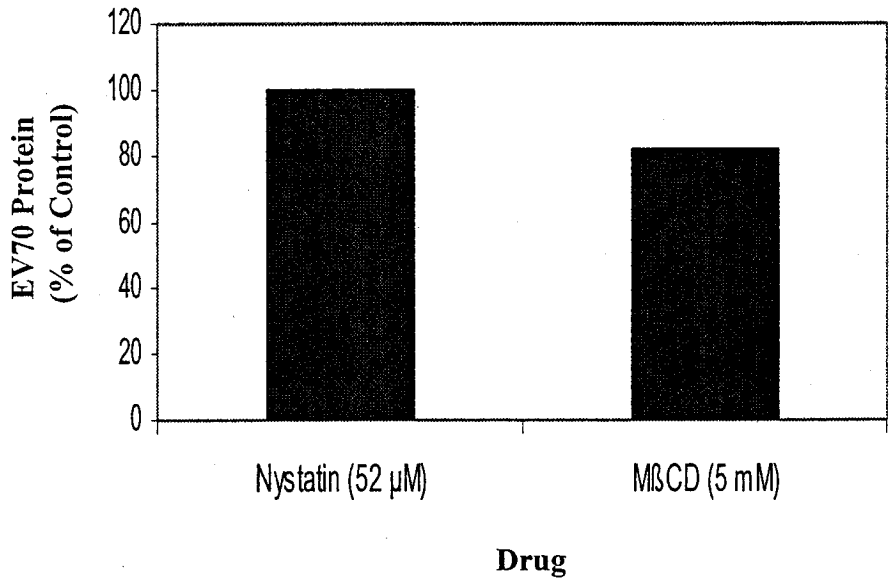
c. Filipin, 10 μM, EV70

d. Nystatin, 104 μM, EV70

Figure 13. Flow cytometry analysis of the effects of cholesterol sequestering agents on EV70 entry into HeLa cells.

HeLa cells growing on 6 well plates were infected with EV70 (MOI=10) following a 30 minute incubation in the presence or absence of either 5 mM MßCD or 52μM of nystatin, at 32 °C. Six hours after infection, in the continued presence of either drug, cells were fixed and permeabilized. EV70 protein synthesis was monitored by intracellular flow cytometry using mAb843 and PE-conjugated goat anti-mouse IgG. Experiment performed once. EV70 protein synthesis, represented as % of control (untreated cells) was calculated as follows:

$$\frac{(\text{MFI treated cells } (1^{\circ}\text{Ab} + 2^{\circ}\text{Ab}))/(\text{MFI treated cells } (2^{\circ}\text{ Ab only})}{(\text{MFI untreated cells } (1^{\circ}\text{Ab} + 2^{\circ}\text{Ab}))/(\text{MFI untreated cells } (2^{\circ}\text{Ab only})} \times 100$$


EV70 Protein
(% of Control)
120
100
80
60
40
20
0
Nystatin (52 µM)
MßCD (5 mM)
Drug

HeLa cells was reduced by treatment with MßCD, and flow cytometry indicated that the EV70 entry was inhibited by approximately 20%, as compared to untreated cells. Together, these results indicated that it is unlikely that EV70 enters cells via a cholesterol-dependent process involving lipid rafts and/or caveolae.

The viability of HeLa cells during incubation in the presence of cholesterol sequestering agents was assessed by trypan blue staining (Figure 11). Six hours of exposure to nystatin concentrations up to 104 μM and 20 hours of exposure to 52 μM nystatin had no effect on cell viability; however, incubation of cells in 10 μM filipin reduced cell viability by 75% within 6 hours.

Because of concerns about the effects of endocytosis inhibitors on other cell functions and because of the variable effects of these drugs on cell viability, it was decided to proceed to studies with dominant-negative mutants of proteins involved in endocytosis, rather than to repeat drug studies several times.

IV. Effects of Dominant-Negative Inhibitors of Endocytosis on EV70 Infection of HeLa Cells.

It was important to use an alternative approach to assess the role of clathrin-mediated endocytosis in entry of EV70 into HeLa cells. Chlorpromazine exerts its effect on clathrin-mediated endocytosis by causing clathrin to localize and accumulate in late endosomes, thereby inhibiting clathrin-coated pit formation (Wang et al., 1993; Orlandi and Fishman, 1998). Chlorpromazine is also known to have effects on other cellular functions such as inhibiting both phosphatidic acid phosphorylase and protein kinase C, and the uncoupling of oxidative phosphorylation (Bachman and Zbinden, 1979; Mori et al., 1980; Pappu and Hauser, 1983). Therefore, it was decided to test dominant-negative

mutants of three proteins involved in endocytosis, eps15, caveolin-1 and dynamin-2, for their effects on EV70 infection of HeLa cells. This approach to elucidating endocytic mechanisms has become increasingly popular in recent years (DeTulleo and Kirchhausen, 1998; Bayer et al., 1999; Pelkmans et al., 2001; Jin et al., 2002; Sieczkarski and Whittaker, 2002b; Marjomaki et al., 2002; Snyers et al., 2003; Chu and Ng, 2004; Querbes et al., 2004; Pietiainen et al., 2004; Chung et al., 2005; Sun et al., 2005; Husain and Moss, 2005; Daecke et al., 2005; Lecot et al., 2005). A total of seven plasmids expressing GFP-tagged wild type (or null) and mutant forms of eps15, caveolin-1 and dynamin-2 were acquired, and transfection conditions were optimized (not shown). Each of the GFP-tagged proteins was readily detected by fluorescent microscopy following transfection of HeLa cells (Figure 14). Caveolin-1 constructs showed a slightly punctate perinuclear fluorescence, dynamin constructs exhibited a very distinctive pronounced punctate pattern and the eps15 constructs showed diffuse cytosolic staining. Sixteen hours after transfection, HeLa cells were infected with EV70 at a MOI of 10, and 6 hours later, viral protein synthesis was analyzed by flow cytometry and immunofluorescent microscopy. As shown in Figure 15, the ratio of double-positive HeLa cells (transfected and infected) to the total population of transfected HeLa cells, expressing either GFP-caveolin-1, which inhibits caveolar endocytosis, or wild type caveolin-1-GFP, were similar, as determined by flow cytometry. Although the ratio of double-positive (transfected and infected) to total transfected cells were low, these results agree with the drug inhibitor experiments and confirm that caveolar endocytosis has little or no role to play in EV70 entry. Figure 16 illustrates that the GFP-tagged DIII eps15 mutant, which has been reported to inhibit clathrin-mediated endocytosis, had an inhibitory effect (25%) on EV70 infection of HeLa cells, as indicated by the decrease in the ratio of double-

Figure 14. Expression of GFP-tagged eps15, caveolin-1, dynamin-2 and their mutants.

HeLa cells growing on LabTek chamber slides were transfected with plasmids encoding the different GFP-tagged proteins using lipofectin. After a 16 hour incubation, GFP-tagged proteins were visualized by fluorescent microscopy at a magnification of 40X. Note that these images have been zoomed in four fold.

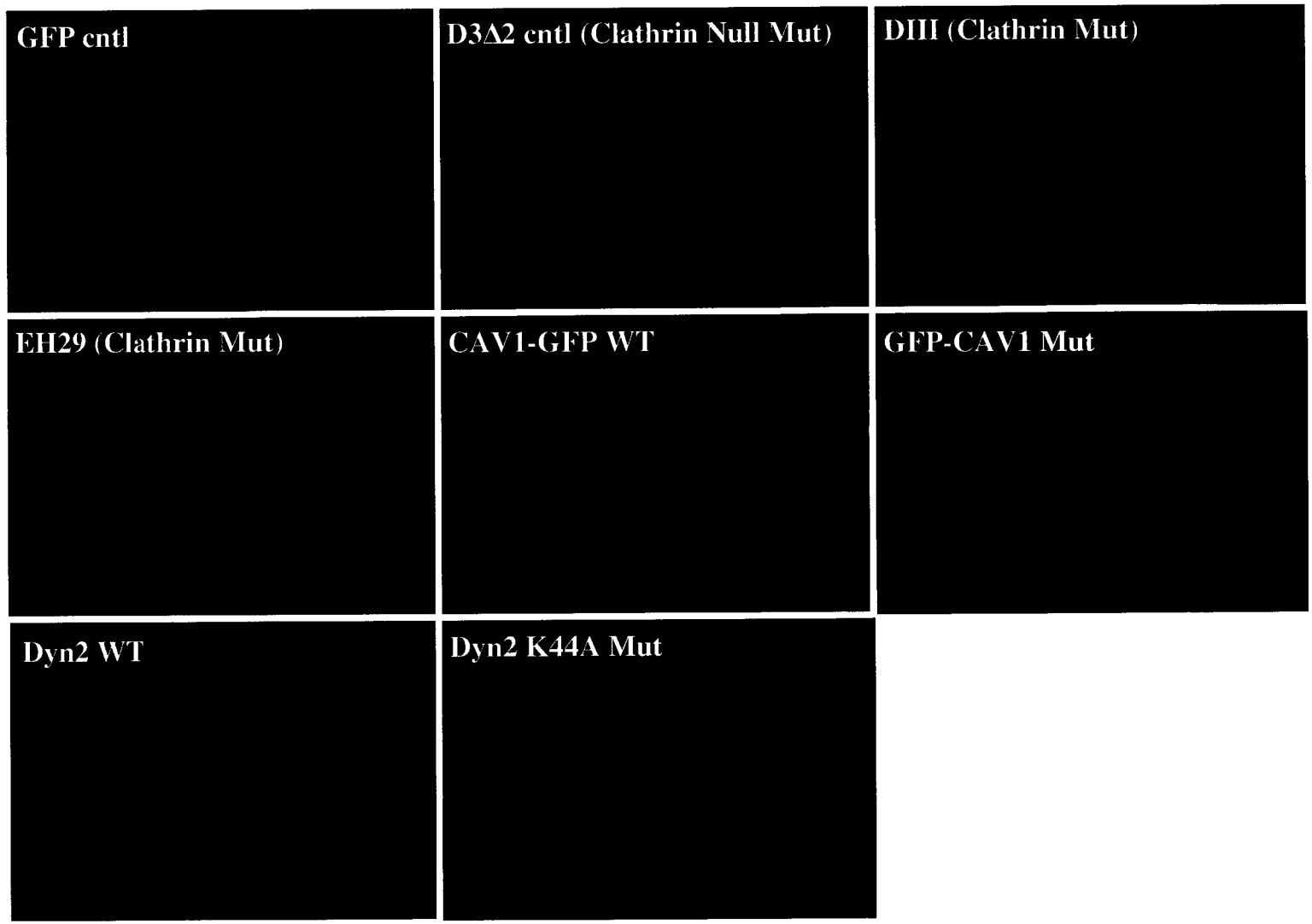
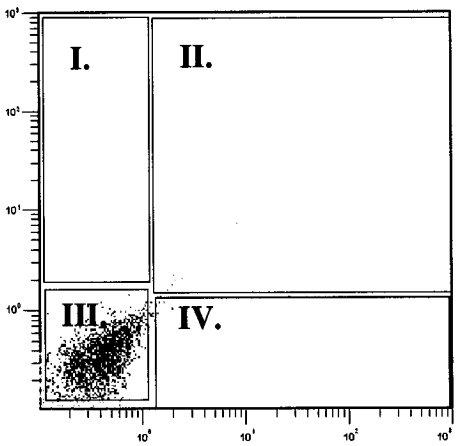

GFP cntl
D3Δ2 cntl (Clathrin Null Mut)
DIII (Clathrin Mut)
EH29 (Clathrin Mut)
CAV1-GFP WT
GFP-CAV1 Mut
Dyn2 WT
Dyn2 K44A Mut

Figure 15. Transfection of HeLa cells with a dominant-negative mutant of caveolin-1 has little or no effect on EV70 entry.

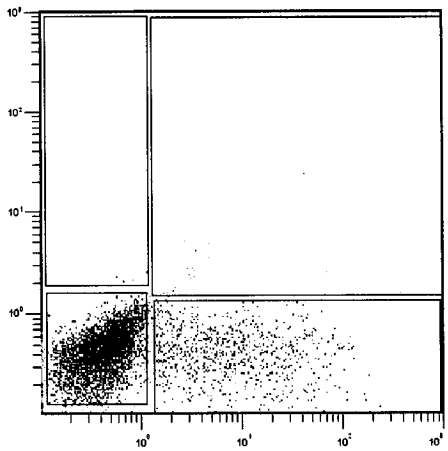
HeLa cells growing in 6 well plates were transfected with plasmids encoding GFP-tagged caveolin-1 (caveolin-1-GFP) or dominant-negative mutant caveolin-1 (GFP-caveolin-1) and incubated at 37 °C. Sixteen hours after transfection, cells were infected with EV70 (MOI=10) at 32 °C. After an additional 6 hours at 32 °C, cells were fixed, permeabilized and EV70 proteins were stained with mAb843 and PE-conjugated goat anti-mouse IgG. Cells were analyzed by flow cytometry. GFP fluorescence was detected in channel 1 (x-axis) and PE fluorescence was detected in channel 2 (y-axis). Data for control cells that were neither transfected or infected are also shown. Quadrant **I** represents cells that are infected but not transfected; quadrant **II** represents cells that are transfected and infected with EV70; quadrant **III** represents untransfected and uninfected cells; and quadrant **IV** represents transfected cells that are not infected. Experiment performed once. Percent in quadrant **II** was calculated as follows:

$$\frac{\text{(Proportion of cells in quadrant II)}}{\text{(Sum of proportion of cells in quadrants II and IV)}} \times 100$$

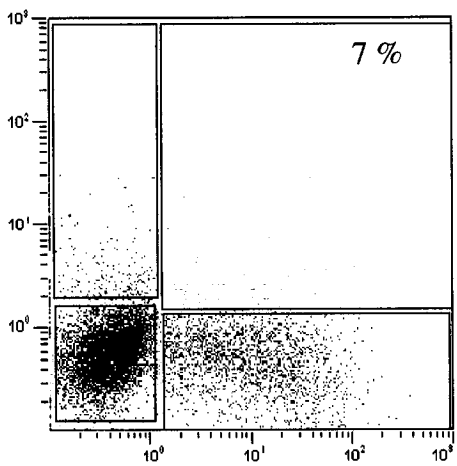
A. No transfection, no infection



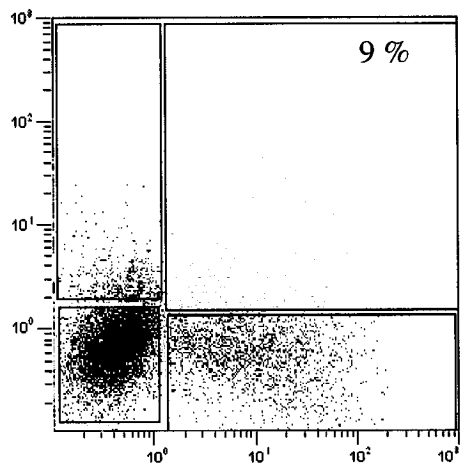
B. GFP-caveolin-1 mutant (no EV70)



C. Caveolin-1-GFP wild type + EV70



D. GFP-caveolin-1 mutant + EV70



Fluorescence in channel 2 (EV70 Protein)

Fluorescence in channel 1 (GFP-tagged Protein)

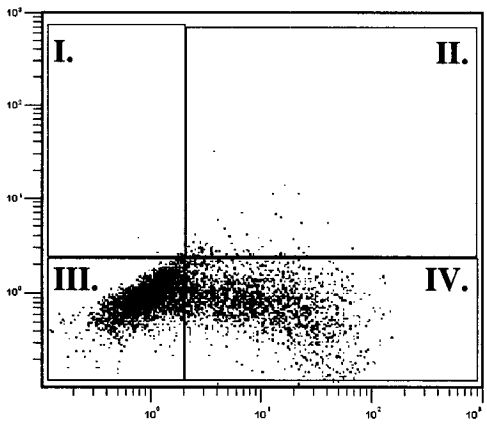
Figure 16. Transfection of HeLa cells with a dominant-negative mutant of eps15 diminishes EV70 entry.

HeLa cells growing in 6 well plates were transfected with plasmids encoding the GFP-tagged null mutant of eps15 (D3Δ2) or the dominant-negative mutant (DIII) and incubated at 37 °C. Sixteen hours after transfection, cells were infected with EV70 (MOI=10) at 32 °C. After an additional 6 hours at 32 °C, cells were fixed, permeabilized and EV70 proteins were stained with mAb843 and PE-conjugated goat anti-mouse IgG. GFP fluorescence was detected in channel 1 (x-axis) and PE fluorescence was detected in channel 2 (y-axis). Data for control cells that were neither transfected or infected are also shown. Quadrant **I** represent cells that are infected but not transfected; quadrant **II** represents cells that are transfected and infected with EV70; quadrant **III** represents untransfected and uninfected cells; and quadrant **IV** represents transfected cells that are not infected. Percent in quadrant **II** was calculated as follows:

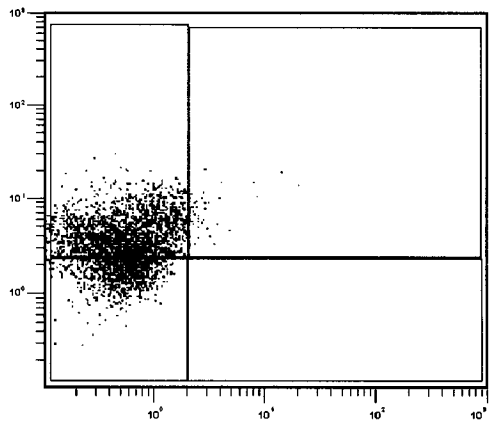
$$\frac{\text{(Proportion of cells in quadrant II)}}{\text{(Sum of proportion of cells in quadrants II and IV)}} \times 100$$

The results are presented as mean percentages ± standard deviation from three independent experiments.

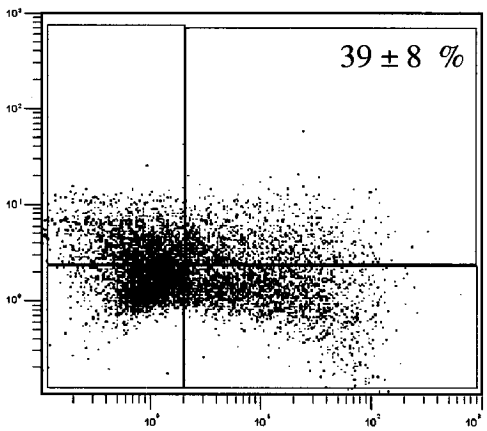
A. Transfected (no EV70)



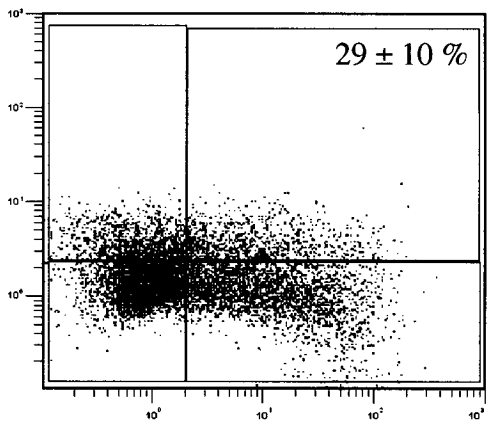
B. Untransfected (EV70)



C. D3Δ2 + EV70



D. DIII + EV70



Fluorescence in channel 2 (EV70 Protein)

Fluorescence in channel 1 (GFP-tagged Protein)

positive cells (transfected and infected) to the total number of transfected cells, as compared to the null mutant of eps15, D3Δ2. Insufficient numbers of infected, GFP-EH29-transfected cells were obtained for flow cytometry analysis, despite several attempts. Expression of DIII in HeLa cells resulted in a 30% decrease in the frequency with which EV70 infected HeLa cells as compared to the null mutant D3Δ2 when analyzed by immunofluorescent microscopy (Table 4). When the effect of EH29 expression in HeLa cells on EV70 infection was analyzed by immunofluorescent microscopy (Table 4), once again, very few EH29-expressing and EV70-infected cells were seen. It is possible that the combined expression of this mutant and infection by EV70 causes these cells to be lost during the experiment. These results are consistent with the idea that EV70 entry into HeLa cells is at least partially clathrin-dependent. As is evident in Figure 17, expression of the GFP-tagged dominant-negative K44A mutant of dynamin-2 had a large inhibitory effect on the ability of EV70 to infect HeLa cells. Most of the cells expressing K44A are not infected by EV70, and most of the infected cells do not express K44A. Inhibition of EV70 infection by K44A was estimated to be approximately 70%, as compared to the wild type control (Table 4). These results suggest that dynamin function is important for EV70 to infect cells. Because dynamin function is necessary for clathrin-mediated endocytosis, this observation is also consistent with a clathrin-dependent process for EV70 entry.

V. EV70 Co-Localizes with Clathrin Light Chains Early After Infection.

Because the experiments with drug and dominant-negative inhibitors of endocytosis suggested that clathrin-mediated endocytosis was important for EV70 entry into HeLa cells, a series of experiments were designed to determine if EV70 co-localizes

Table 4. EV70 relies on dynamin-dependent endocytosis to enter HeLa cells.

HeLa cells growing on LabTek chamber slides were transfected with plasmids encoding GFP-tagged versions of dynamin-2, or its dominant-negative mutant, K44A, or the null mutant of eps15, D3Δ2, or dominant-negative mutants, DIII or EH29, and incubated at 37 °C. Sixteen hours after transfection, cells were infected with EV70 (MOI=20) at 32 °C. After an additional 6 hours incubation at 32 °C, cells were fixed, permeabilized and EV70 proteins were stained with mAb843 and Alexa555-conjugated goat anti-mouse IgG. GFP-tagged proteins and Alexa555-stained EV70 proteins were visualized by fluorescent microscopy at a magnification of 40X. The number of GFP-positive cells (transfected) and Alexa555-positive cells (EV70-infected) in 5-7 randomly chosen fields were counted for each experiment. Percent inhibition was calculated as follows:

$$\left[1 - \frac{(\text{GFP\&EV70 +ve cells for mutant}) / (\text{GFP +ve cells for mutant})}{(\text{GFP\&EV70 +ve cells for WT}) / (\text{GFP +ve cells for WT})}\right] \times 100$$

Results are presented as mean percentages ± standard deviation from three independent experiments. P value < 0.05 for K44A and DIII but $P > 0.05$ for EH29.

Construct	Percent Inhibition (relative to control) ± standard deviation	(GFP+EV70 +ve cells) /(total transfected cells)
K44A (Dynamin Mutant)	70.9 ± 13.5	33 / 315
DIII (Clathrin Mutant)	29.7 ± 14.7	90 / 439
EH29 (Clathrin Mutant)	20.6 ± 53.5	10 / 53

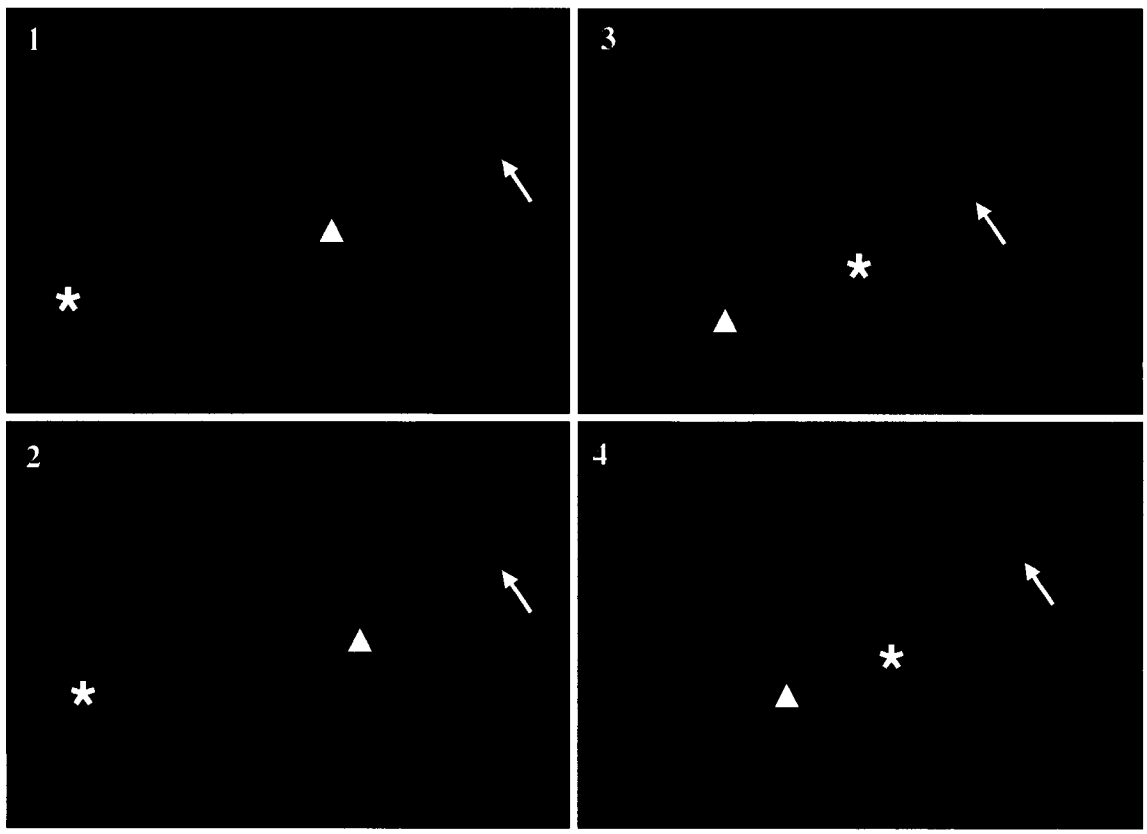
Figure 17. Transfection of HeLa cells with a dominant-negative mutant of dynamin-2 inhibits EV70 entry.

A and B. HeLa cells growing on LabTek chamber slides were transfected with plasmids encoding GFP-tagged versions of dynamin-2 (**A**, panels **1-4**) or its dominant-negative mutant, K44A (**B**, panels **5-10**) and incubated at 37 °C. Sixteen hours after transfection, cells were infected with EV70 (MOI=20) at 32 °C. After an additional 6 hours incubation at 32 °C, cells were fixed, permeabilized and EV70 proteins were stained with mAb843 and Alexa555-conjugated goat anti-mouse IgG. GFP-tagged dynamin-2 (green) and EV70 proteins (red) were visualized by fluorescent microscopy, and brightfield images were also collected, at a magnification of 40X. In A, arrows, arrowheads, and asterisks represent different cells. Panels **1** and **2** represent the same field, as do panels **3** and **4**. In B, the arrows and asterisks represent different cells. Panels **5-7** represent the same field, as do panels **8-10**. Note all images have been zoomed in four fold.

A.

I. Dynamin wild type

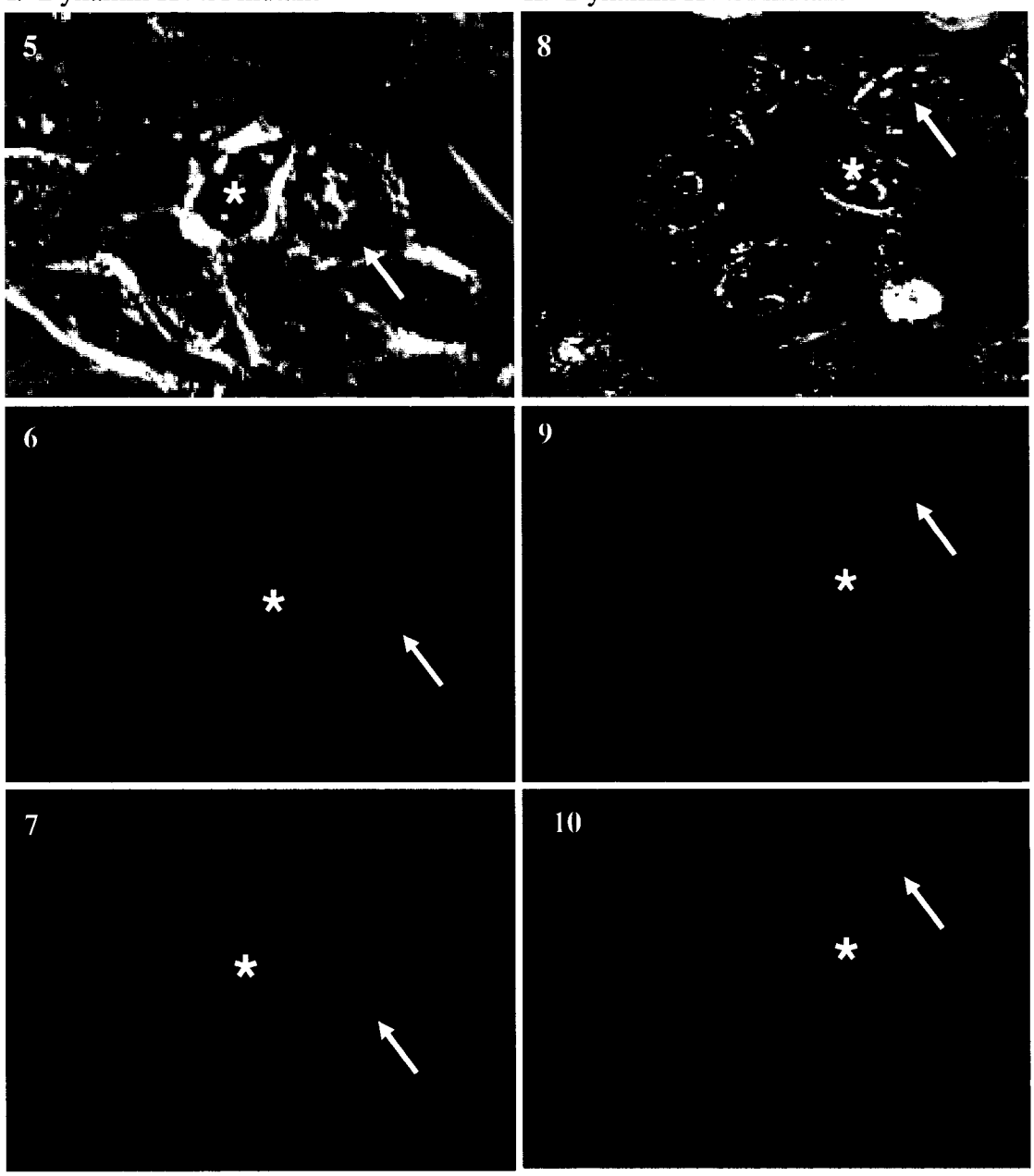
II. Dynamin wild type



B.

I. Dynamin K44A mutant

II. Dynamin K44A mutant



with clathrin. Others have used co-localization of markers of endocytosis with viral particles as support for a virus using a particular type of entry pathway (Joki-Korpela et al., 2001; Triantafilou and Triantafilou, 2004; Pietiainen et al., 2004; Chung et al., 2005; Berryman et al., 2005). EV70 conjugated with Alexa555 was provided by Dr. Reza Nokhbeh (Department of Biochemistry, Microbiology and Immunology, University of Ottawa) and allowed to bind to HeLa cells at 4 °C. Cells were shifted to 32 °C to allow virus entry to begin and then examined by immunofluorescent microscopy at different times after the temperature shift. As shown in Figure 18, immediately after binding, diffuse fluorescent "speckles", presumably representing fluorescent virus particles, were present on the periphery of cells. By 20 minutes after the temperature shift, the number of speckles had diminished somewhat. By 1 hour after the temperature shift, a punctate pattern of larger fluorescent areas were seen throughout the cytoplasm, suggesting that EV70 had entered cells. By 3 hours after the temperature shift, these large fluorescent areas appeared to be localized in the perinuclear region, a pattern that may represent concentration of fluorescent virus particles in a specific cellular compartment. At later time points fluorescence decreased, presumably due to degradation of virus particles. These results suggested that it would be feasible to test whether or not Alexa555-tagged EV70 would co-localize with clathrin during virus entry.

HeLa cells were transfected with a plasmid expressing EYFP-tagged clathrin light chain. Sixteen hours after transfection, cells were incubated with Alexa555-labelled EV70 and then examined at different times after infection by immunofluorescent microscopy. As shown in Figure 19, at time 0, and as described above, Alexa555-tagged virus was located on the outer surfaces of cells. Clathrin light chain was present surrounding the cell surface and throughout the cytoplasm. One hour after infection,

Figure 18. Time course of infection of HeLa cells with Alexa555-labelled EV70.

HeLa cells growing on LabTek chamber slides were incubated with Alexa555-labelled EV70 (~0.25 μg) for one hour at 4 °C to allow binding. Cells were transferred to 32 °C to initiate virus entry, and at various times after transfer, cells were fixed with paraformaldehyde. Virus was visualized by fluorescent microscopy at 40X magnification. Experiment performed once.

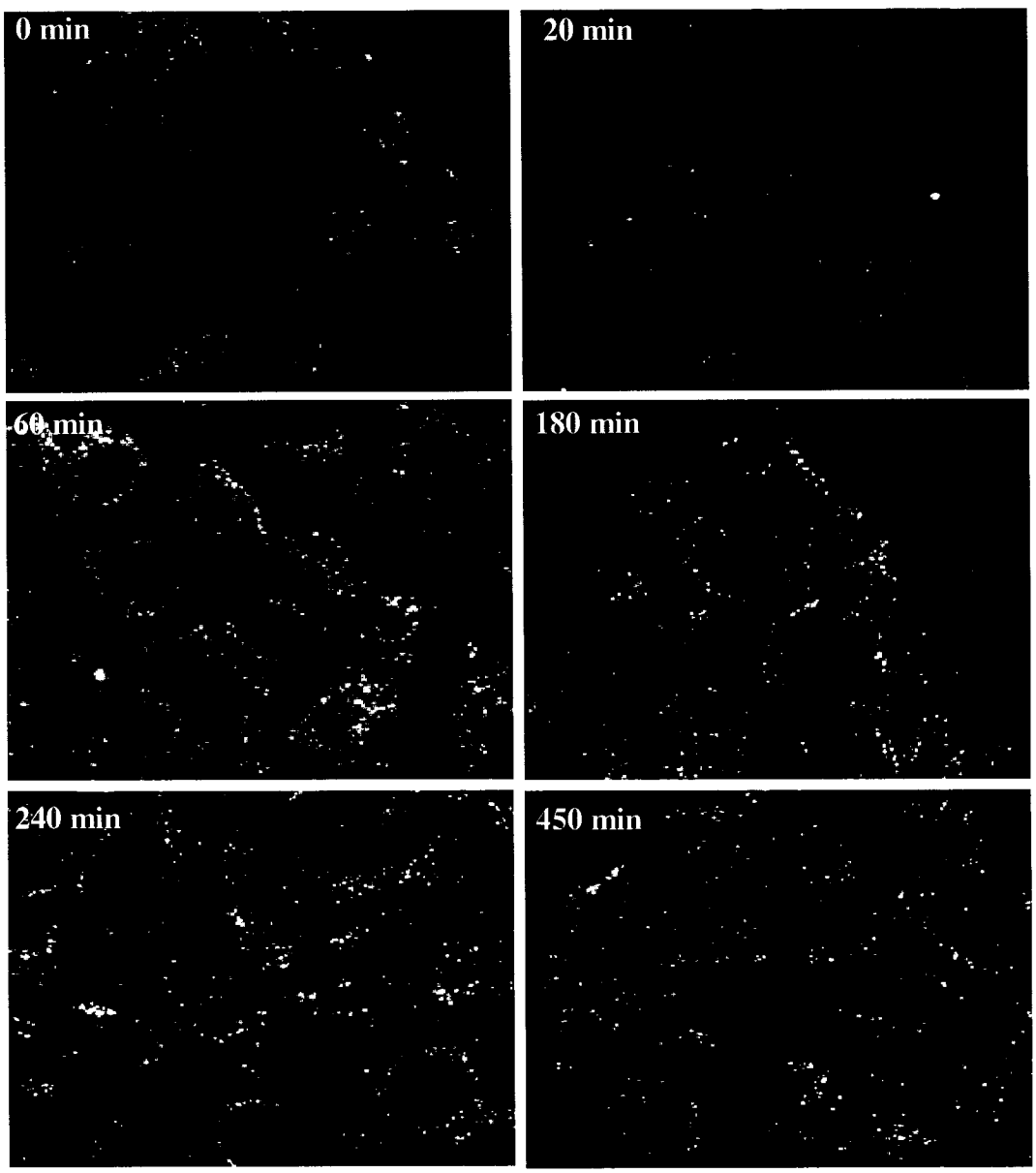
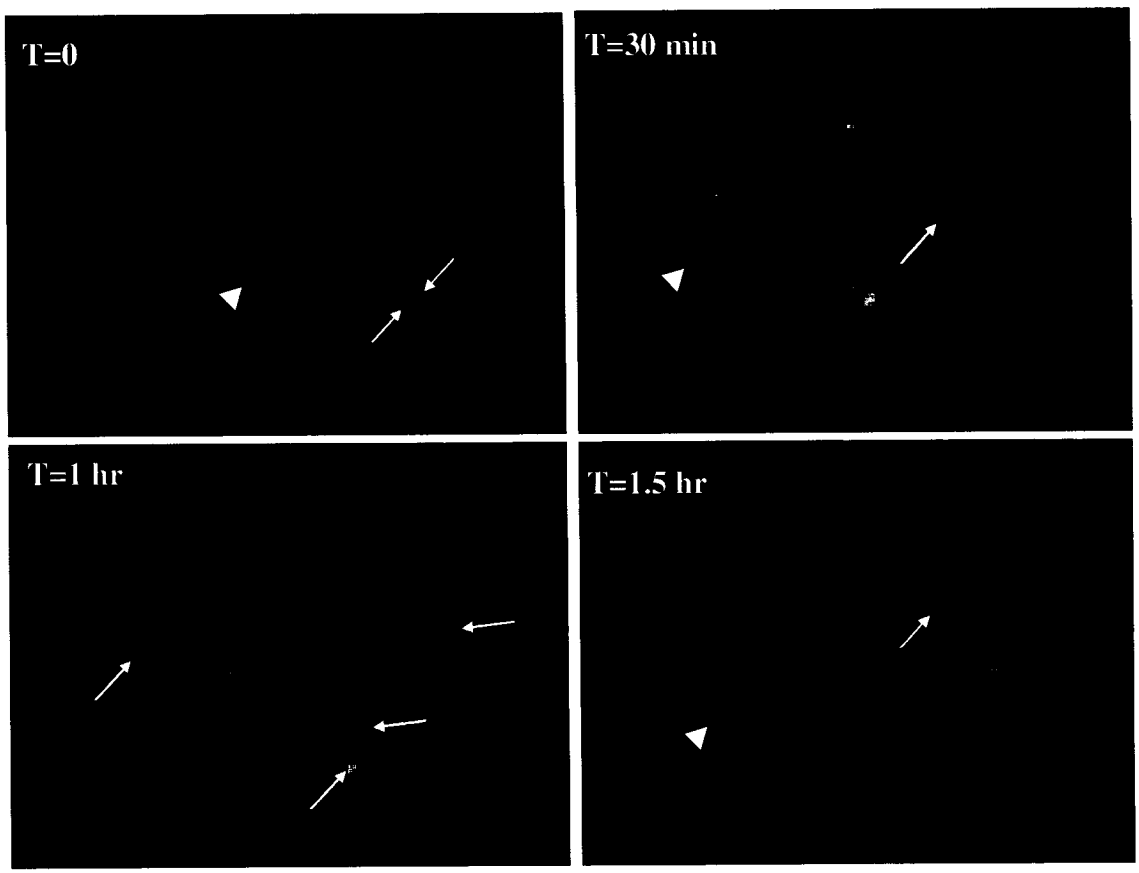

0 min
20 min
60 min
180 min
240 min
450 min

Figure 19. Co-localization of EYFP-tagged clathrin light chain and Alexa555-labelled EV70.

HeLa cells growing on LabTek chamber slides were transfected with plasmid DNA encoding EYFP-tagged clathrin light chain. Sixteen hours after transfection, cells were incubated with Alexa555-labelled EV70 (~0.25 μg) for one hour at 4 °C to allow binding. Cells were transferred to 32 °C to initiate virus entry and at various times after transfer, cells were fixed with paraformaldehyde. EV70 (red) and clathrin light chain (green) were visualized by fluorescent microscopy at 40X magnification. Arrowheads represent Alexa555-labelled EV70 (red) and arrows represent co-localization of EV70 and clathrin light chain (orange). Note that images are zoomed in 10 fold. Nuclei were stained with Hoechst dye (0.1 μg/mL). Experiment performed once.


T=0
T=30 min
T=1 hr
T=1.5 hr

EV70 had entered the cytosol and, in the merged image, some of the virus appears to be co-localized with clathrin light chain, as indicated by arrows pointing to orange spots. This suggests that EV70 is entering the cytoplasm in close proximity to clathrin light chain, presumably in clathrin-coated pits and vesicles. At later times, the frequency of virus and clathrin light chain co-localization is much reduced , presumably because virus is no longer associated with clathrin light chain.

Together, the results obtained with inhibitors of endocytosis and the co-localization studies are consistent with the hypothesis that clathrin-mediated endocytosis plays a major role in EV70 infection of HeLa cells.

DISCUSSION.

The overall objectives of the research presented in this thesis were to demonstrate that EV70 employs endocytosis to enter HeLa cells and to characterize the endocytic pathway that EV70 uses to infect HeLa cells. The first experimental objective in this research was to establish an intracellular flow cytometry protocol to detect EV70 protein synthesis in HeLa cells. Intracellular flow cytometry was chosen over the traditional plaque assay to allow for more rapid detection of infected cells and to minimize the effects of drugs or other treatments on cell viability. This objective was accomplished; EV70 proteins could be reliably detected in infected HeLa cells within 6 hours of infection. Immunofluorescence methods that identify EV70-infected cells in the same time frame were also developed during the course of the research. The appearance of newly synthesized EV70 protein in HeLa cells was used as an indicator that virus had entered cells and initiated the infectious cycle.

Effects of Drugs That Inhibit Endocytosis on EV70 Entry.

As a first step to determining if EV70 utilizes endocytosis for infection of HeLa cells, several drugs that inhibit either clathrin-mediated or lipid raft- and caveolin-dependent endocytosis were tested for their ability to interfere with EV70 entry. Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, reduced EV70 infection of HeLa cells by almost 60%, as determined by flow cytometry, and inhibition was also seen by immunofluorescent microscopy. Chlorpromazine reduced the yield of EV70 as well but this may not be due to direct effects on EV70 entry, since cell viability, assessed by trypan blue exclusion, was also compromised during the long exposure to chlorpromazine required for these experiments. Chlorpromazine treatment also inhibited

the endocytosis of Tf, a marker for clathrin-mediated endocytosis (Ghosh et al., 1994; Johannes and Lamaze, 2002). Chlorpromazine is classified as a CAD which all share two distinct domains: a hydrophobic (often aromatic) domain and an ionisable nitrogen atom which can become positively charged (Liscovitch and Lavie, 1991). Chlorpromazine inhibits clathrin-mediated endocytosis because it prevents the proper recycling of components of clathrin-coated pits, such as AP-2. AP-2 is required for clathrin-coated pit formation since it is a binding molecule that targets clathrin to the cellular surface (Wang et al., 1993). Chlorpromazine reverses an on/off switch that controls AP-2 binding to cellular membranes; thus AP-2 can no longer bind to cellular membranes, a required step for clathrin triskelions to target and bind to membranes through the interaction with AP-2 (Wang et al., 1993).

Cholesterol sequestering agents such as filipin and nystatin, which inhibit lipid raft- and caveolin-mediated endocytosis had only small effects on the ability of EV70 to infect cells; though it is noted that EV70 protein synthesis decreased in the presence of filipin when measured by flow cytometry, but no inhibition was seen when measured by plaque assay or by immunofluorescent microscopy. A method to resolve this would be to repeat the flow cytometry data for filipin. MßCD, which binds cholesterol in its hydrophobic pocket and depletes cellular membranes of cholesterol (Rothberg et al., 1992; Neufeld et al., 1996; Christian et al., 1997; Orlandi and Fishman, 1998), reduced EV70 infection of HeLa cells by approximately 20 % and this inhibition of EV70 protein synthesis was also visible by immunofluorescent microscopy. Cholesterol depletion by MßCD could affect virus entry by affecting membrane functions, as indicated by its effects on cellular signalling pathways. MßCD treatment of several cell lines, such as Cos-1 cells and NIH-3T3 cells, results in the activation of mitogen-activated protein

kinase pathways through the activation of phosphatidylinositol-3 kinase (Chen and Resh, 2001). It is possible that altering membrane functions, including signalling pathways, by MßCD treatment could affect EV70 entry into HeLa cells. Uptake of CTxB was assessed by fluorescent microscopy, and was used to confirm that lipid raft- and caveolin-mediated entry processes were inhibited by cholesterol sequestering agents. CTxB is endocytosed through the caveolin-mediated route in most cells types that have been analyzed, however, it has been reported that a clathrin-mediated pathway may account for a small proportion of CTxB uptake in cells that express low levels of caveolin-1, such as in HeLa cells (Schapiro et al., 1998; Singh et al., 2003). Uptake of CTxB was partially inhibited by nystatin and filipin but a dramatic reduction was seen with MßCD. Flow cytometry was not used to follow CTxB uptake since no fluorescence was detected by flow despite repeated attempts, and thus was abandoned. Treatment of HeLa cells with the cholesterol sequestering drugs had no effect on virus yield although filipin greatly reduced cell viability. These results support the idea that lipid raft- or caveolin-dependent entry of EV70 are unlikely, although the observation that MßCD partially inhibits EV70 infection of HeLa cells and the observation that filipin inhibits EV70 when measured by flow cytometry suggests that the potential contribution of a cholesterol-dependent route cannot be ruled out entirely. However, the results with MßCD may also be consistent with a role for clathrin-mediated endocytosis in EV70 entry. Several laboratories have claimed that MßCD can inhibit both clathrin- and caveolin-mediated endocytic routes at high concentrations. Snyers and colleagues (2003) showed that 10 mM MßCD inhibited endocytosis of human rhinovirus 2, which is recognized as entering cells via a clathrin-dependent route. By electron microscopy, Rodal and colleagues (1999) found that MßCD removed both invaginated caveolae and clathrin-coated pits from the cell surface of HEp-

2 cells and that endocytosis of Tf was inhibited 50% in HeLa cells treated with 10 mM MßCD. Although MßCD has been shown to have a possible effect on clathrin-mediated processes, nystatin and filipin do not (Orlandi and Fishman, 1998).

Because only chlorpromazine inhibited EV70 infection of HeLa cells to any great extent, the results of experiments with drug inhibitors of endocytosis strongly suggested that EV70 enters cells via a clathrin-mediated mechanism.

Dominant-Negative Mutants and Endocytosis.

The results from drug inhibition studies suggested that EV70 exploits a clathrin-mediated endocytic route to enter HeLa cells. While drugs are commonly used to inhibit endocytosis, they are recognized to have pleiotropic effects (Bishop, 1998; Subtil et al., 1999; Rodal et al., 1999; Sieczkarski and Whittaker, 2002a). CADs like chlorpromazine also affect a number of other cellular enzymes and processes. CADs, including chlorpromazine have been shown to inhibit protein kinase C, with an IC_{50} value of 30 μM (Mori et al., 1980; Naghshineh et al., 1986), Ca^{2+}/Calmodulin-dependent enzymes, with an IC_{50} value of 10 μM (Wess and Wallace, 1980; McNaughton et al., 2001), Na^+, K^+-ATPase, with an IC_{50} value of 1 μM (Akera T and Brody, 1970; Muller et al., 1991) and to uncouple oxidative phosphorylation, with an IC_{50} value of 300 μM (Bachman and Zbinden, 1979; Tsao et al., 1982).

Therefore, the results obtained with the drug inhibitors and co-localization experiments needed confirmation by other methods. Dominant-negative mutants of components of endocytic pathways have been used to elucidate the endocytic mechanisms of a number of viruses (DeTulleo and Kirchhausen, 1998; Bayer et al., 1999; Pelkmans et al., 2001; Jin et al., 2002; Sieczkarski and Whittaker, 2002b; Marjomaki et

al., 2002; Snyers et al., 2003; Chu and Ng, 2004; Querbes et al., 2004; Pietiainen et al., 2004; Chung et al., 2005; Sun et al., 2005; Lecot et al., 2005; Husain and Moss, 2005; Daecke et al., 2005).

It was decided to test the effects of the expression of dominant-negative mutants of eps15, caveolin-1 and dynamin-2 in HeLa cells on EV70 infection. Dominant-negative mutants exert their effects by becoming part of and thereby inactivating the function of protein complexes. This approach was considered to be a way to provide more specific information about the EV70 entry process. Plasmids encoding GFP-tagged mutant and wild type constructs of eps15, caveolin-1 and dynamin-2 were transfected into HeLa cells and, once expression of the GFP-tagged proteins occurred, the cells were infected with EV70 and analyzed by flow cytometry and/or fluorescent microscopy. Expression of the dynamin-2 mutant, K44A, in HeLa cells decreased EV70 infection by 70%. The clathrin mutant, DIII, caused a 25% and 30% reduction in EV70 infection as compared to HeLa cells expressing a null mutant, D3Δ2, by flow cytometry and fluorescent microscopy, respectively. A second clathrin mutant, EH29, also appeared to inhibit EV70 entry but the low numbers of cells transfected with this plasmid make these data less reliable. The caveolin-1 mutant had no effect on EV70 entry.

The results of the experiments with dominant-negative inhibitors, therefore: (1) confirm that caveolin-mediated processes are unlikely to be involved, to any degree, in EV70 entry into HeLa cells; (2) indicate that dynamin-2 function is essential for EV70 infection; and (3) are consistent with the chlorpromazine inhibition results that point to a clathrin-mediated pathway.

Not all transfected cells expressed proteins at an equal level; the variable expression of GFP-tagged proteins of endocytosis may have had differential effects on EV70 infection.

Co-Localization of Clathrin and EV70.

A number of laboratories have used co-localization of markers of endocytosis with viral particles as support for a virus using a particular type of entry pathway (Joki-Korpela et al., 2001; Triantafilou and Triantafilou, 2004; Pietiainen et al., 2004; Chung et al., 2005; Berryman et al., 2005). Given that chlorpromazine inhibited EV70 infection of HeLa cells and dominant-negative inhibitors indicated that caveolin-mediated endocytosis is unlikely to be involved in EV70 uptake into HeLa cells; experiments were performed to determine if EV70 co-localized with clathrin light chain, a component of clathrin-coated pits and vesicles. Entry of Alexa555-labelled EV70 into HeLa cells expressing EYFP-tagged clathrin light chain was examined by fluorescent microscopy, at different time intervals. EV70 and clathrin light chain appeared to co-localize in a few loci, presumably clathrin-coated pits or vesicles, within 1 hour after infection. Although only a few sites of co-localization were identified, these results are consistent with the idea that EV70 enters HeLa cells by a clathrin-dependent mechanism. Co-localization studies can be better addressed by confocal microscopy since the use of a laser as opposed to a UV bulb, as in fluorescent microscopy, allows the user to excite fluorescent molecules in a very thin plane within a cell. The kinetics of clathrin-mediated entry have been determined for several viruses. Influenza virus, human rhinovirus 2 and JC virus are released from clathrin-containing structures within minutes of entry (Pho et al., 2000; Sieczkarski and Whittaker, 2002b; Snyers et al., 2003; Lakadamyali et al., 2004; Nunes-Correia et al.,

2004; Rust et al., 2004). Therefore, it may be necessary to look earlier than 30 minutes after infection to detect co-localization of EV70 viral particles with clathrin light chains. For one virus, however, the kinetics of clathrin-mediated endocytosis have been shown to be quite prolonged. Bovine papilloma virus 1 (BPV-1) has been shown to enter cells by clathrin-mediated endocytosis, but the kinetics of internalization were unusually slow, with the half life of entry of BPV-1 being approximately 4 hours versus 5-15 minutes for a typical ligand (Day et al., 2003).

Data and some issues of uncertainty in the results can be resolved by repeating some of the experiments, particularly some of the flow analyses. Analyzing larger number of cells by fluorescent microscopy would reduce experimental error and improve statistical power. In some experiments, EV70 infection appeared to be lower in cells transfected with wild type GFP-tagged proteins of endocytosis than for non-transfected cells. It is possible that transfecting agents can adversely affect the ability of EV70 to infect cells by perturbing the plasma membrane. Lipofectin has been suggested to have a nonspecific, detergent-like effect upon the cell membrane (Yeoman et al., 1992) and to have some detrimental effects on viruses or cells (Innes et al., 1990). Lipofectamine may have similar effects. This may be resolved by using alternative methods for introducing plasmids into cells, such as nucleofection (or electroporation), which require no "lipofecting" agents.

Approaches for using siRNA to inhibit endocytosis have been developed, and should provide an alternative methodology for untangling virus entry mechanisms. Examples of siRNA approaches include elucidating the endocytic pathway in equine infectious anemia virus, HIV-1, VSV, adenovirus, and coxsackievirus B (Chen et al., 2005; Rose et al., 2005; Sun et al., 2005; Meier et al., 2005; Yuan et al., 2005). The half

lives of some components of endocytic pathways can be quite long and the functional redundancy of coated-pit proteins, their homologues and their multiple isoforms must be considered (Sorkin, 2004). For instance, depletion of epsin-1 did not significantly influence clathrin-mediated endocytosis, but this may be due to functional redundancy with epsin-2 (Sorkin, 2004; Huang et al., 2004). Another component involved in clathrin-mediated endocytosis is Rab5a, where the elimination of a combination of three Rab5 isoforms (Rab5a, Rab5b, and Rab5c) by siRNA is necessary to inhibit clathrin-mediated endocytosis (Huang et al., 2004).

Picornaviruses employ a variety of different entry mechanisms. Some examples of picornaviruses that use clathrin-mediated routes include human rhinoviruses 2 and 14, FMDV, HPEV-1, and coxsackievirus B3 (DeTulleo and Kirchhausen, 1998; Joki-Korpela et al., 2001; Snyers et al., 2003; Chung et al., 2005; O'Donnell et al., 2005). Other picornaviruses, such as echoviruses 1 and 11, and coxsackieviruses A9 and B4, make use of caveolin-mediated endocytosis (Stuart et al., 2002; Marjomaki et al., 2002; Triantafilou and Triantafilou, 2003; Triantafilou and Triantafilou, 2004). Poliovirus may enter cells via a clathrin-, caveolin-, and dynamin-independent route (DeTulleo and Kirchhausen, 1998; Kronenberger et al., 1998; Danthi and Chow, 2004). Some picornaviruses appear to follow more than one route. Echovirus 11 was shown to enter via both lipid raft-mediated endocytosis as well as a clathrin-mediated endocytic route (Stuart et al., 2002) and echovirus 1, which uses a caveolin-mediated entry route, may also use a dynamin- and lipid raft-dependent mechanism (Marjomaki et al., 2002).

One would expect that the nature of a viral receptor would have a major influence on endocytosis of viral particles. FMDV has been shown to utilize at least four members of the α subgroup of the integrin family of receptors. O'Donnell and colleagues (2005)

showed that FMDV particles co-localize with a marker of clathrin-coated pits but not to markers of caveolin-mediated endocytosis. They also showed that viral replication begins due to acidification of endocytic vesicles. HPEV-1 utilizes the α5 integrins as internalization receptors and has also been shown to use clathrin-mediated endocytosis for entry (Joki-Korpela et al., 2001). These data suggest that the nature of the viral receptor may determine the pathway of entry. However, echovirus 1 also makes use of an integrin, α2ß1, but appears to enter cells by a caveolin- and lipid raft-dependent mechanism (Marjomaki et al., 2002; Pietiainen et al., 2004). Partial co-localization of echovirus 1 with SV40 and CTxB, demonstrated that intracellular virus-containing vesicles were caveosomes (Pietiainen et al., 2004). However, the same dominant-negative mutant of caveolin-1 used in the studies presented in this work, GFP-caveolin-1, had no effect on echovirus 1 entry and infection. This suggests the possibility that a parallel, non-caveolar entry mechanism that is dynamin- and lipid raft-dependent, may exist (Pietiainen et al., 2004). Coxsackievirus A9 utilizes integrin α5ß3 as a receptor and glucose regulated protein 78 as a co-receptor; the latter molecule appears to associate with a MHC class I molecule that is responsible for virus internalization via a lipid raft-dependent mechanism (Triantafilou and Triantafilou, 2003). In summary, at least four different picornaviruses utilize integrin receptors to infect cells but there is no common entry process.

Even picornaviruses that engage the same receptor may be internalized by different mechanisms. A particularly relevant example is that provided by coxsackieviruses B3 and B4. Both of these viruses bind to CD55 and utilize CAR as an entry molecule. Coxsackievirus B3 enters cells through clathrin-coated pits (Chung et al., 2005) but coxsackievirus B4 entry is via lipid rafts and is clathrin-independent

(Triantafilou and Triantafilou, 2004). How each of these viruses interacts with CD55 or the co-receptor may determine which endocytosis pathway is triggered. CD55 is the major attachment molecule for EV70 infection of HeLa cells (Karnauchow et al., 1996; Alexander and Dimock, 2002; Haddad et al., 2004; Nokhbeh et al., 2005), and is a common component of lipid rafts. However, even this knowledge does not help identify which endocytic pathway EV70 might use to infect HeLa cells. It is not known if EV70 requires a co-receptor for cell infection.

The following proposal is suggested: that the major mechanism exploited by EV70 to enter HeLa cells is a dynamin- and clathrin-dependent, but caveolin-independent endocytic process. EV70 may also use an alternative entry pathway that requires dynamin, but is clathrin- and caveolin-independent. There are examples of other viruses for which such an entry process has been proposed, although these routes are not well characterized. Echovirus 1, as described above, may use such a mechanism as an alternative route to its major caveolar route (Marjomaki et al., 2002). Similarly, influenza virus appears to enter cells via a clathrin- and caveolin-independent endocytic pathway as an alternative route to its normal clathrin-dependent process (Lakadamyali et al., 2004; Nunes-Correia et al., 2004). Rotavirus also exploits a clathrin- and caveolin-independent, but dynamin-dependent pathway (Lopez and Arias, 2004).

A compelling question to ask is: "Does EV70 use different endocytic routes in different cell lines?" Investigating the entry process in cell lines other than HeLa cells might prove informative. EV70 recognizes different receptors on other cell lines such as human corneal epithelial cells and U937 cells, where the major EV70 receptor on corneal epithelial cells is a sialylated, non-GPI anchored protein, while sialylated receptors on U-937 cells are not proteinaceous (Nokhbeh et al., 2005). On HeLa cells, CD55 is the main

binding molecule (Karnauchow et al., 1996; Alexander and Dimock, 2002). Thus, EV70 appears to be able to interact with at least three different cell surface molecules on three different cell lines. An interesting proposition, as presented earlier, is to determine whether a common entry mechanism exists among the different cell lines which EV70 infects or does it vary from cell line to cell line, as has been demonstrated for other viruses (e.g. mouse polyomavirus (Richterova et al., 2001; Gilbert et al., 2003), Epstein-Barr virus (Miller and Hutt-Fletcher, 1992), human cytomegalovirus (Bodaghi et al., 1999), herpes simplex virus (Nicola et al., 2003), murine leukemia virus (Kizhatil and Albritton, 1997).

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