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# RECEPTORS AND ENDOCYTOSIS OF COXSACKIEVIRUS A9

by

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To Tommi, Oona and Elmo

If I held in my hand
Every grain of sand
Since time first began to be
Still, I could never count
Measure the amount
Of all the things you are to me
(Secret Garden)

# ABSTRACT

Outi Heikkilä
**Receptors and endocytosis of coxsackievirus A9**
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Coxsackievirus A9 (CV-A9) belongs to human enteroviruses within family *Picornaviridae*, which are the main cause of aseptic meningitis. In addition, CV-A9 causes a wide range of other clinical manifestations of acute disease including respiratory infections, myocarditis, encephalitis and severe generalized infections in newborns.

In this study, the functions of integrins αVβ6 and αVβ3 in the attachment and cellular entry of CV-A9 were analyzed. Further, virus and cell surface interactions and endocytosis of CV-A9 were studied in specific cell lines. Also, a method for production of GFP-expressing CV-A9 particles by long PCR-mediated mutagenesis and *in vivo* transcription was developed.

The results indicated that RGD-motif (arginine-glycine-asparagine) that resides in the viral capsid is important for CV-A9 infection particularly in cell lines expressing integrin αVβ6 and that this integrin serves as a high affinity attachment receptor for the virus. CV-A9 is also capable of infecting certain cell lines independently of αV-integrins by binding to the cell surface HSPA5 protein. Regardless of the attachment stage, the internalization of the virus occurs via the same entry pathway and is dependent on β2M, dynamin, and Arf6 but independent of clathrin and caveolin-1. Furthermore, the virus internalization occurs within Arf6-containing vesicles suggesting that Arf6 is central mediator of CV-A9 endocytosis. While in this study the results of CV-A9 endocytosis were based on microscopical visualization within individual fixed cells, a rapid method for generation of a virus for real-time imaging was also described.

**Keywords:** coxsackievirus A9, integrin, RGD-motif, endocytosis, dynamin, HSPA5, Arf6, β2-microglobulin, GFP

# TIIVISTELMÄ

Outi Heikkilä
**Coxsackie A9 -viruksen reseptorit ja kulkeutuminen solussa**
Virusoppi, Turun yliopisto, Turku
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Coxsackievirus A9 (CV-A9) on *Picornaviridae*-heimoon kuuluva enterovirus. Se on yleinen ihmisen taudinaiheuttajavirus, joka lievien nuhakuumeiden lisäksi aiheuttaa mm. aseptista aivokalvontulehdusta, hengitystieinfektioita, sydänlihastulehduksia, aivotulehdusta sekä vakavia infektioita vastasyntyneille.

Tässä väitöskirjatyössä tutkittiin kahden eri αV-integriinin, αVβ6 ja αVβ3, roolia coxsackievirus A9:n sitoutumisessa solun pintaan. Lisäksi työssä syvennyttiin integriinien merkitykseen CV-A9:n infektiossa, tutkittiin viruksen ja solunpinnan välisiä vuorovaikutuksia sekä seurattiin viruksen endosytoosia eli kulkeutumista solun pinnalta solun sisäpuolelle ja edelleen kohti solun keskusta kahdessa eri isäntäsolulinjassa. Työssä kehitettiin myös PCR-menetelmä, jonka avulla tuotettiin GFP:tä ilmentäviä CV-A9-partikkeleita *in vivo* -transkriptiolla.

Tulokset osoittivat, että coxsackievirus A9:n pinnalla sijaitseva RGD-alue (arginiini-glysiini-asparagiini) on tärkeä virusinfektiossa silloin, kun isäntäsolun pinnalla ilmennetään αVβ6-integriiniä. Lisäksi osoitettiin, että αVβ6 toimii viruksen ensisijaisena integriinireseptorina, sillä se sitoutuu virukseen voimakkaammin kuin αVβ3-integriini. CV-A9 pystyy kuitenkin infektoimaan joitakin solulinjoja ilman integriinejä sitoutumalla solunpinnan HSPA5-proteiiniin. Riippumatta solun molekyyleistä, joiden avulla virus sitoutuu solun pintaan, virus kulkeutuu solun sisään samalla mekanismilla, jossa keskeisiä molekyylejä ovat β2-mikroglobuliini, dynamiini ja Arf6. Viruksen havaittiin myös kulkeutuvan solun sisäpuolella Arf6-molekyylejä sisältävässä vesikkelissä, minkä vuoksi Arf6 vaikuttaa olevan keskeinen tekijä CV-A9:n infektiossa. Tässä työssä CV-A9:n endosytoosia seurattiin yksittäisissä soluissa tietyissä aikapisteissä. Jatkotutkimuksia varten väitöskirjatyössä kehitettiin nopea menetelmä, jolla tuotettiin fluoresoivaa proteiinia ilmentäviä CV-A9-partikkeleita työkaluiksi virusinfektion kuvantamiseen monisoluisissa organelleissa, kuten kudoksissa ja koe-eläimissä.

**Avainsanat:** coxsackievirus A9, integriini, RGD-alue, endosytoosi, dynamiini, HSPA5, Arf6, β2-mikroglobuliini, GFP

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# ABBREVIATIONS

| | |
|---|---|
| A549 | human epithelial lung carcinoma cell line |
| Arf | ADP-ribosylation factor |
| β2M | β2-microglobulin |
| CAV1 | caveolin-1 |
| CCV | clathrin-coated vesicle |
| CME | clathrin-mediated endocytosis |
| CNS | central nervous system |
| CV | coxsackievirus |
| CV-A9 | coxsackievirus A9 |
| DN | dominant-negative |
| E | echovirus |
| EE | early endosome |
| EIPA | 5-(*N*-ethyl-*N*-isopropyl)amiloride |
| ER | endoplasmic reticulum |
| ERC | endosomal recycling compartment |
| EV | enterovirus |
| FLIM | fluorescence-lifetime imaging |
| FMDV | foot-and-mouth disease virus |
| GFP | green fluorescent protein |
| GMK | African green monkey kidney cell line |
| HPeV | human parechovirus |
| HRP | horseradish peroxidase |
| HSPA5 | Hsp70 protein of the endoplasmic reticulum; also known as immunoglobulin heavy chain binding protein (BiP) and glucose regulated protein-78 (GRP78) |

| | |
|---|---|
| HSPG | heparan sulfonated proteoglycan cell surface attachment factor |
| LE | late endosome |
| MAb | monoclonal antibody |
| ME | maturing endosome |
| MHC | major histocompatibility complex |
| m.o.i | multiplicity of infection |
| ORF | open reading frame |
| PEV | primary endocytic vesicle |
| Pfu | plaque forming unit |
| PI3K | phosphatidyl-inositol 3-kinase |
| PV | poliovirus |
| RD | human rhabdomyosarcoma cell line |
| RE | recycling endosome |
| RGD | arginine-glycine-asparagine tripeptide |
| siRNA | small interfering RNA |
| STED | stimulated emission depletion microscopy |
| SW480 | human epithelial colorectal adenocarcinoma cell line |
| WT | wild type |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:

**I** **Heikkilä**, **O**., Susi, P., Stanway, G. & Hyypiä, T. (2009). Integrin αVβ6 is a high-affinity receptor for coxsackievirus A9. Journal of General Virology **90**: 197-204.

**II** **Heikkilä**, **O**., Susi, P., Tevaluoto, T., Härmä, H., Marjomäki, V., Hyypiä, T. & Kiljunen, S. (2010). Internalization of coxsackievirus A9 is mediated by β2-microglobulin, dynamin and Arf6 but not caveolin or clathrin. Journal of Virology **84**: 3666-3681.

**III** **Heikkilä, O**., Karelehto, E., Merilahti, P., Tevaluoto, T., Sukki, M., Alanko, J., Kiljunen, S., Himanen, J.-P. & Susi, P. (2013). HSPA5 and β2-microglobulin target coxsackievirus A9 to dynamin-dependent Arf6 entry pathway in human epithelial colon adenocarcinoma cell line. Submitted to Journal of Virology.

**IV** **Heikkilä**, **O**., Kainulainen, M., Susi, P. (2011). A combined method for rescue of modified enteroviruses by mutagenic primers, long PCR and T7 RNA polymerase-driven in vivo transcription. Journal of Virological Methods **171**: 129-133.

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# 1 INTRODUCTION

Viruses are small pathogens that need the host cell for proliferation. They attach to the cell surface via specific "house-hold" proteins, receptors, and use them to penetrate the plasma membrane. Viruses utilize cellular transport systems, which carry the "cargo" from the cell surface to the interior and vice versa. Inside the cell, viral genome is released to produce viral progeny that can then be released from the cell interior to initiate a new infection cycle. Although some viruses penetrate the cytosol directly through the plasma membrane, most are dependent on endocytic uptake and vesicular transport, which takes place in the cytoplasm. The most studied endocytic routes are clathrin-mediated endocytosis (CME), caveolin/raft-dependent endocytosis and macropinocytosis (reviewed by Mercer and Helenius, 2012, Mercer et al., 2010 and Doherty et al., 2009). Recently, several clathrin- and caveolin/raft-independent mechanisms have been identified but they remain poorly characterized.

The family *Picornaviridae* consists of a large number of small RNA viruses including entero-, rhino-, polio-, echo- and coxsackieviruses, and foot-and-mouth disease virus, which cause a wide spectrum of diseases in humans and livestock. Virus transmission occurs mainly through fecal-oral and respiratory routes. Although picornaviruses are the most common human viruses and both clinically and economically important (Ehrenfeld et al., 2010, Whitton et al., 2005, Fendrick et al., 2003), there are no approved drugs for therapy.

In this thesis, the receptor use and mechanisms of endocytosis of coxsackievirus A9 (CV-A9) were studied. CV-A9 is a significant human pathogen that belongs to the *Enterovirus B* species in the family *Picornaviridae*. Like many enteroviruses, it causes respiratory infections and more severe diseases such as infections in central nervous system (meningitis and encephalitis) and myocarditis. Recent outbreaks of aseptic meningitis and exanthematous disease in children caused by CV-A9 have been reported in China 2005 (Cui et al., 2010) and Japan 2012 (Aoki et al., 2012). In addition, CV-A9 has been associated with type 1 diabetes (Roivainen et al., 1998, Hyöty et al., 1995) indicating the clinical importance of the virus.

The aims of this thesis were to study the role of integrin receptors in CV-A9 infection using three different human cell lines, epithelial lung carcinoma (A549), rhabdomyosarcoma (RD) and epithelial colorectal adenocarcinoma (SW480). In addition, the mechanisms of endocytosis of CV-A9 were studied in A549 and SW480 cells. Finally, a time-saving method for generation of GFP-expressing CV-A9 particles was developed, which may be useful in real-time imaging of CV-A9 infection in tissues and animal models.

# 2 REVIEW OF LITERATURE

## 2.1 Picornavirus taxonomy and human diseases

The family *Picornaviridae* currently consists of seventeen genera; *Aphtovirus*, *Aquamavirus*, *Avihepatovirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Megrivirus*, *Parechovirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus* (Knowles et al., 2012, ICTV Master Species List 2012 v1), which are further divided into 37 species and numerous serotypes (**Table 1;** The Picornavirus Study Group: www.picornastudygroup.com, The Picornavirus Home Page: picornaviridae.com and International Committee on Taxonomy of Viruses, ICTV: www.ictvonline.org).

Previously, the classification of picornaviruses was based on their pathogenesis in laboratory animals or cultured cells but today they are mainly classified into genera and species according to genetic information (Ehrenfeld et al., 2010, Hyypiä et al., 1997). Generally, viruses are classified as follows (picornaviruses are used as an example): Order: *Picornavirinae*; Family: *Picornaviridae*; Genus: e.g. *Enterovirus*; Species: e.g. *Enterovirus B*; Type: e.g. coxsackievirus A9; Isolate or Strain: identified by GenBank acc no. All levels except types and isolates are written with capital letter and in cursive. Due to adoption of genetic methods in virus typing, the number of picornavirus types has increased by 40% during the past fifteen years (Knowles et al., 2012), which has complicated virus classification. Lack of efficient typing methods has also hampered studies on disease associations. Thus, the large number of virus types and insufficient detection methods are still the main reasons why it has been difficult to develop control measures against picornaviruses; there are no approved drugs against picornaviruses and only three of them (poliovirus, hepatitis A virus and animal foot-and-mouth disease virus, FMDV) can be controlled by vaccination.

The genus *Enterovirus* is one of the eight picornavirus genera containing virus types that infect humans albeit the majority of infections are mild or asymptomatic. It is important to notice that in the current classification scheme this genus contains both entero- and rhinoviruses although they have rather different pathogenic features. The genus *Enterovirus* includes twelve species, and seven of them are typical human pathogens: human rhinoviruses (*Rhinovirus A*, *B* and *C* species) are common cold viruses, and they have also been suggested to be the causative agents of wheezing and asthma exacerbation (Kim and Gern 2012, Kotaniemi-Syrjänen et al., 2003) whereas human enteroviruses (*Enterovirus A*, *B*, *C* and *D* species) are typical among children causing wide spectrum of diseases such as respiratory illnesses, aseptic meningitis, hand-, foot- and mouth-disease, rash, myocarditis and paralytic disease.

Symptoms of hand-, foot- and mouth-disease are mainly associated with two virus types, coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-71), in the *Enterovirus A* species. The characteristic disease symptoms, which are generally mild, are headache, fever, and

sore throat including blisters on throat, palms, hands and feet. However, EV-71 is also associated with severe infections of the central nervous system. Recent EV-71 outbreaks have occurred in Malaysia (Ooi et al., 2007), Taiwan (Chen et al., 2007), Singapore (Chan et al., 2003), Brunei (AbuBakar et al., 2009), Thailand (Chatproedprai, et al., 2010), Korea (Ryu et al., 2010), Hong Kong (Ma et al., 2010) and in different parts of China (Mao et al., 2010, Cao et al., 2010, Li et al., 2005). Interestingly, the European isolates have not generally been epidemic, which has been explained by genetic differences between European and Asian viruses (Bible et al., 2007).

*Enterovirus B* species (EV-B) is the largest picornavirus species, and includes coxsackieviruses (types B1-B6; CV-Bs), echoviruses and numbered enteroviruses that affect the central nervous system and heart, being responsible for numerous clinical manifestations such as encephalitis, meningitis and myocarditis. Despite the name coxsackievirus A9 (CV-A9) also belongs to EV-B species (see below). The most extensively studied enterovirus is poliovirus (PV) that belongs to the species *Enterovirus C*. Poliovirus is still a problem in some parts of the world regardless of the vaccination campaign that aims to eradicate the disease; according to World Health Organization (WHO), Afghanistan, Nigeria and Pakistan remained polio endemic in 2012 (Poliomyelitis fact sheet, WHO). Polio infects mainly children under five years of age and the initial symptoms of the disease are fever, fatigue, headache, vomiting, stiffness in the neck and pain in the limbs. The infection can lead to irreversible paralysis and 5-10% of paralyzed patients die of shortness of breath.

*Enterovirus D* species contain serotypes associated with respiratory infections (enterovirus 68; Oberste et al., 2004) and acute hemorrhagic conjunctivitis (enterovirus 70; Mirkovic et al., 1973). Particularly in the last few years, the reports of enterovirus 68 (EV-68) have dramatically increased (Lauinger et al., 2012) and the virus type has been associated with severe infections of respiratory tract (Rahamat-Langendoen, 2011, Meijer et al., 2010) and central nervous system (Kreuter et al., 2011). Although it is possible that the increased EV-68 findings are due to the improved typing, Lauinger et al. (2012) highlight the possibility that EV-68 is an emerging pathogen that requires global monitoring. However, the most commonly detected enterovirus serotypes in Europe are CV-A16, CV-A9, CV-B2-5 and echoviruses 6, 7, 9, 11, 13, 19 and 30 (Antona et al., 2007, Maguire et al., 1999, Hovi et al., 1996). Furthermore, infections with coxsackievirus A9 and coxsackieviruses B1-B6 are associated with the pathogenesis of type 1 diabetes (T1D, Tauriainen et al., 2011, Roivainen et al., 1998, Hyöty et al., 1995).

Other picornavirus genera that contain virus types being capable of infecting humans are *Aphtho-*, *Cardio-*, *Cosa-*, *Hepato-*, *Kobu-*, *Parecho- and Saliviruses*. Although *Aphtho-*, *Cardio-*, and *Kobuviruses* are mainly animal viruses, they all include species that can use human as their host such as *Equine rhinitis A virus* (ERAV; *Aphthovirus* genus; Plummer, 1963), *Theilovirus* (genus *Cardiovirus*; Liang et al., 2008) and *Aichivirus A* (genus *Kobuvirus;* Yamashita et al., 1998). Aichiviruses were first discovered in 1989 during the outbreak of oyster-associated gastroenteritis (Yamashita et al., 1991) and the virus seems to be circulating

worldwide including Finland (Kaikkonen et al., 2010). Recently, Aichivirus infections in elderly people have also been reported in Sweden (Jonsson et al., 2012). *Cosavirus* and *Salivirus* have just recently been classified as new picornavirus genera (ICTV: http://www.ictvonline.org) and they both include virus type/types which have been reported to cause gastroenteritis and therefore acute diarrhea (Li et al., 2009, Holtz et al., 2008).

**Table 1.** The current classification of picornaviruses includes 17 genera and 37 species.

| Genus | Species | Examples of types |
|---|---|---|
| *Aphthovirus* | *Bovine rhinitis A virus* | |
| | *Bovine rhinitis B virus* | |
| | *Equine rhinitis A virus* | equine rhinitis A virus 1 |
| | *Foot-and-mouth disease virus* | |
| *Aquamavirus* | *Aquamavirus A* | |
| *Avihepatovirus* | *Duck hepatitis A virus* | |
| *Cardiovirus* | *Encephalomyocarditis virus* | |
| | *Theilovirus* | Vilyuisk human encephalomyelitis virus (VHEV) |
| *Cosavirus* | *Cosavirus A* | cosavirus A |
| *Dicipivirus* | *Cadicivirus A* | |
| *Enterovirus* | *Enterovirus A* | coxsackievirus A16, enterovirus A71 |
| | ***Enterovirus B*** | **coxsackievirus A9**, coxsackieviruses B1-B6 |
| | *Enterovirus C* | poliovirus |
| | *Enterovirus D* | enteroviruses 68 and 70 |
| | *Enterovirus E* | |
| | *Enterovirus F* | |
| | *Enterovirus G* | |
| | *Enterovirus H* | |
| | *Enterovirus J* | |
| | *Rhinovirus A* | |
| | *Rhinovirus B* | |
| | *Rhinovirus C* | |
| *Erbovirus* | *Equine rhinitis B virus* | |
| *Hepatovirus* | *Hepatitis A virus* | hepatitis A virus 1 |
| *Kobuvirus* | *Aichivirus A* | Aichi virus 1 |
| | *Aichivirus B* | |
| | *Aichivirus C* | |
| *Megrivirus* | *Melegrivirus A* | |
| *Parechovirus* | *Human parechovirus* | human parechoviruses 1, 2 and 3 |
| | *Ljungan virus* | |
| *Salivirus* | *Salivirus A* | salivirus A |
| *Sapelovirus* | *Avian sapelovirus* | |
| | *Porcine sapelovirus* | |
| | *Simian sapelovirus* | |
| *Senecavirus* | *Seneca Valley virus* | |
| *Teschovirus* | *Porcine teschovirus* | |
| *Tremovirus* | *Avian encephalomyelitis virus* | |

* The genera containing human pathogens are underlined.
* Coxsackievirus A9 belongs to *Enterovirus B* species and is marked in bold.
* International Committee on Taxonomy of Viruses, ICTV: http://www.ictvonline.org

The clinical symptoms of human parechoviruses (HPeV) vary from mild respiratory and gastrointestinal infections to severe diseases in central nervous system (CNS) and sepsis-like syndromes. The infections caused by serotypes HPeV-1, HPeV-2 and recently found HPeV-3 exist in early childhood, typically before the age of three years (Benchop et al., 2006, Joki-Korpela and Hyypiä, 2001). HPeV-1 and HPeV-2 are mainly associated with mild diseases while HPeV-3 is the cause of serious CNS infections particularly among newborns (Westerhuis et al., 2012, Kolehmainen et al., 2012, Benchop et al., 2006). The genus *Hepatovirus* includes only a single species, *Hepatitis A virus* (HAV). The virus is transmitted through contaminated water and seafood causing the outbreaks of acute hepatitis. Although the disease is usually asymptomatic in young children, the risk of acute liver failure and death increases with age and with the presence of chronic liver disease (Franco et al., 2012, Keeffe, 2006). In Finland, the incidence of hepatitis A has been very low for decades due to increased hygiene excluding the outbreaks in 1994-1995 and 2002-2003, which were connected to intravenous drug use and, despite increased travel throughout the world, the rate of incidence is continuously decreasing due to an effective vaccine that became available in 1992 (Broman et al., 2010, Kuusi et al., 2003, Leino et al., 1997).

## 2.2 Properties of picornaviruses

### 2.2.1 Particle structure

Picornavirus particle is small and non-enveloped, about 30 nm in diameter. Several picornavirus capsid structures have been resolved in recent years including polioviruses (Filman et al., 1989, Hogle et al., 1985), foot-and-mouth disease virus (Acharya et al., 1989), coxsackievirus B3 (Muckelbauer et al., 1995), echovirus 1 (Filman et al., 1998), **coxsackievirus A9** (Hendry et al., 1999), rhinoviruses 2 and 14 (Verdaguer et al., 2000, Rossmann et al., 1985), echovirus 11 (Stuart et al., 2002) and enterovirus 71 (Plevka et al., 2012). All resolved capsid structures are highly similar consisting of 60 protomers each of which contains four structural capsid proteins VP1 to VP4 or exceptionally three capsid proteins VP0, VP1 and VP3 in the case of human parechoviruses (Stanway et al., 1994, Hyypiä et al., 1992). In a single protomer unit (**Figure 1**) VP1, VP2 and VP3 form the icosahedral shell whereas VP4, a small protein generally with an N-terminal myristic acid modification, resides on the inner surface of the particle in close vicinity to the viral genome (Racaniello, 2007, Tesar et al., 1993, Chow et al., 1987). VP1-3 proteins fold into similar “jelly roll”-structure containing wedge-shaped eight-stranded β-barrel in which the β-strands are connected with loops that vary in length and composition in different picornavirus types. These loops and the tilts of the β-barrel together determine the receptor binding specificity and antigenic properties of the virus. VP1 components are located around the fivefold axes of symmetry while VP2 and VP3 alternate around the two- and threefold axes (Ehrenfeld et al., 2010). Five protomers form a pentagon-shaped pentamer, and twelve pentamers form a complete icosahedral capsid of the virus.

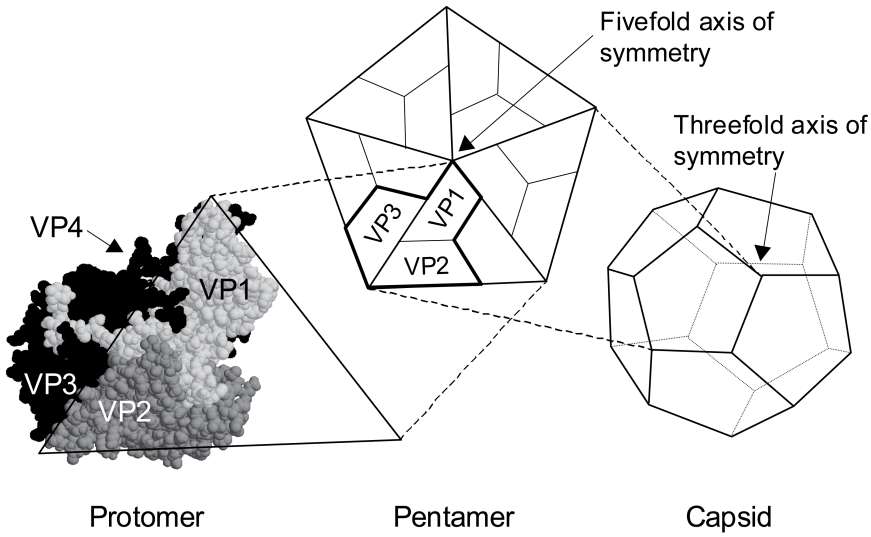


**Figure 1.** The capsid structure of picornaviruses. One protomer consists of the four capsid proteins VP1 to VP4 and five protomers form a single pentamer. Twelve pentamers together form the viral icosahedral capsid. (Modified from Airaksinen, 2000).

### 2.2.2 Genome organization

Picornavirus genome is a single-stranded positive-sense RNA molecule consisting of approximately 7000-8800 nucleotides (**Figure 2A**) with a median of about 7600 bases (Ehrenfeld et al., 2010). The genome itself is infectious and acts as a messenger RNA in a host cell. Across the picornavirus family, the genome organization is similar but not identical (Racaniello, 2007). A small virus-encoded protein VPg (Viral Protein of the genome) is covalently linked to the 5' terminus via a tyrosine hydroxyl and phosphodiester bond (**Figures 2A** and **B**) and it serves as a protein primer for initiation of viral RNA synthesis (Paul et al., 1998). The 3' end of the genome is polyadenylated (Poly(A)) in all picornaviruses but the length varies among virus species (Ehrenfeld et al., 2010). A single open reading frame (ORF) encoding viral capsid (VP1-4) and replication proteins (2A-C and 3A-D) resides between the two untranslated regions, 5'UTR and 3'UTR. The 5'UTR is the most complex structural region of the picornavirus genome bearing several motifs that include the species-specific terminal domain. In enteroviruses, the domain takes the form of a cloverleaf-shaped motif (5'CL in **Figure 2B**) while all other genera have unbranched terminal stems varying in length (Ehrenfeld et al., 2010, Andino et al., 1990). In addition, the most dominant structural unit with branched and unbranched stems in the 5' UTR is the internal ribosome entry site (IRES) that promotes polyprotein translation (Hellen and de Breyne, 2007, Pelletier and Sonenberg, 1988, Jang et al., 1988). The 3' UTR region has also highly organized secondary structure elements that include a varying number of stem loops depending on virus genus and type (Serrano et al., 2006, Jacobson et al., 1993, Pilipenko et al., 1992). Although the 3' UTR has been implicated in both translation and replication together with the poly(A) tract, the exact functional role of the 3' UTR is still unclear (Kok

et al., 2012, Merkle et al., 2002, Todd et al., 1997). The loosely paired unbranched regions called CREs (Cis-active RNA elements) are the most recently described picornavirus RNA structures (McKnight and Lemon, 1998). They reside in the 5' UTR, 3' UTR and ORF; the exact locations depend on the virus genus and species (Steil and Barton, 2009). Almost all found CREs have been shown to be essential for RNA replication excluding the CREs in parechoviruses and species C rhinovirus, which have not been experimentally tested yet (Al-Sunaidi et al., 2007, Gerber et al., 2001).

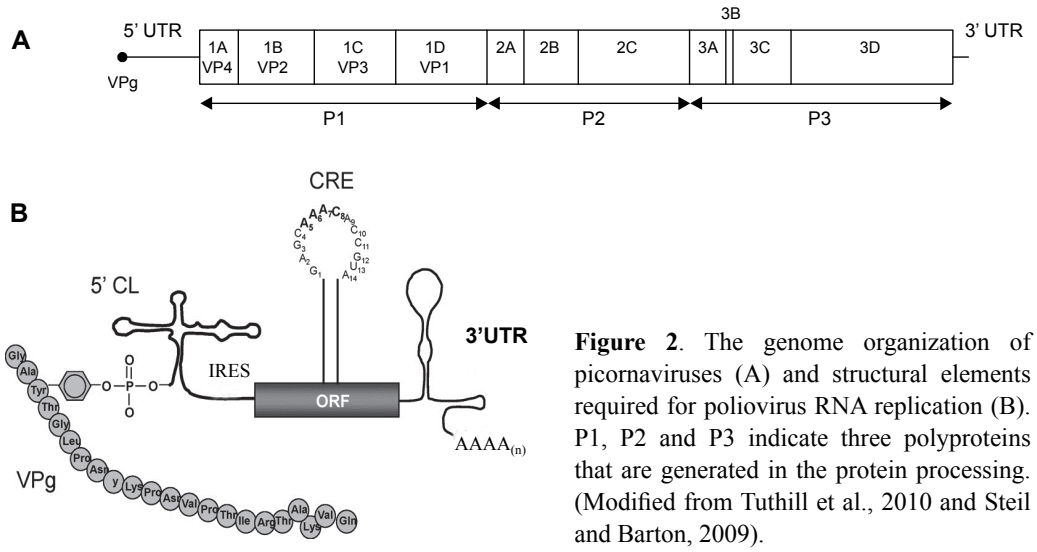


**Figure 2**. The genome organization of picornaviruses (A) and structural elements required for poliovirus RNA replication (B). P1, P2 and P3 indicate three polyproteins that are generated in the protein processing. (Modified from Tuthill et al., 2010 and Steil and Barton, 2009).

## 2.3 Infection cycle of picornaviruses

The picornavirus infection cycle begins with the attachment of the virus to a cell surface receptor (**Figure 3,** step 1). The receptor specificity, which determines the cell and tissue tropism and therefore pathogenesis, varies depending on picornavirus genera and species (Tuthill et al., 2010, Rossmann et al., 2002). By now more than ten cell surface receptors for picornaviruses have been identified including the members of the immunoglobulin-like family, the low density lipoproteins receptor (LDLR) family, the complement control family, the integrin family of cell adhesion molecules and the T cell immunoglobulin domain mucin-like domain receptors. Virus-receptor interaction triggers viral uptake into the cell through a specific endocytic vesicle (**Figure 3,** step 2) that depends on the virus type, receptor specificity and cell line. Clathrin- (CME) and caveolin-1- (CAV1) mediated pathways are the best known endocytosis mechanisms for picornaviruses (Kim and Bergelson, 2012, Berryman et al., 2005, Pietiäinen et al., 2004, Marjomäki et al., 2002, Joki-Korpela et al., 2001, DeTulleo and Kirchhausen, 1998) but there are also many other less characterized (CME- and CAV1-independent) endocytic mechanisms (Khan et al., 2011, Heikkilä et al., 2010 (II), Brandenburg et al., 2007). During recent years, the complexity of endocytic mechanisms has become increasingly evident. Virus uncoating occurs during vesicular transport leading to the

release of picornaviral RNA genome. The viral RNA is translated into a polyprotein that is then autocleaved into the structural and non-structural proteins (**Figure 3**, steps 3 and 4). With the help of viral-encoded non-structural proteins positive- and negative-sense RNA strands are synthesized in the membrane-associated replication complexes that reside on the outside surface of replication vesicles (**Figure 3**, steps 5 and 6), and finally, new virus particles are assembled and released (**Figure 3**, steps 7 and 8).

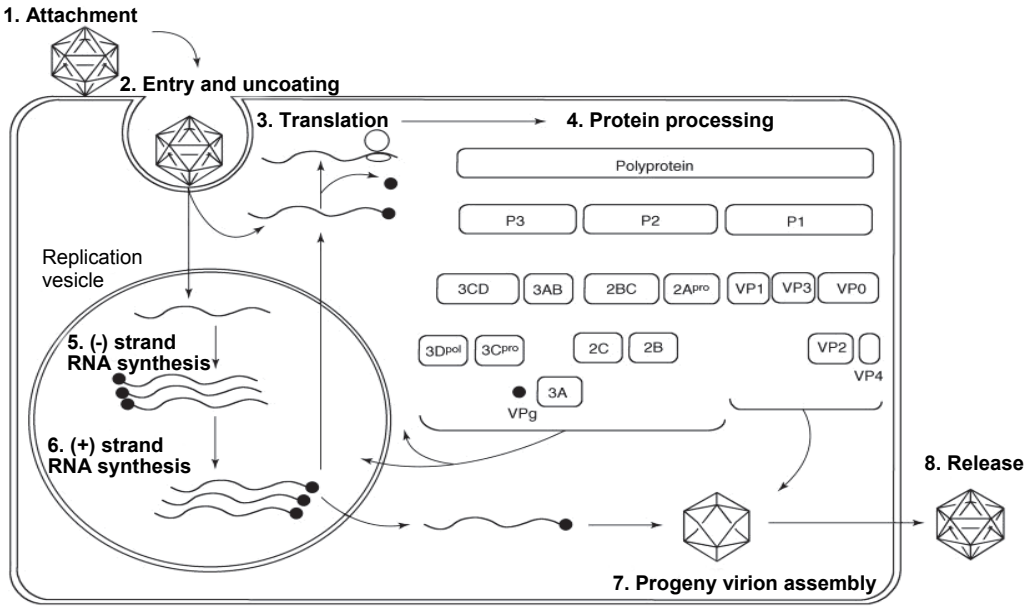


**Figure 3.** The infection cycle of picornaviruses in 8 steps: (1) particle attachment to a host cell surface via a cellular receptor, (2) entry, uncoating and genome release, (3) viral genome translation, (4) protein processing into the nonstructural and structural proteins, (5 and 6) viral RNA replication in the replication vesicle, (7) virion assembly and (8) particle release (Modified from Wu et al., 2010).

### 2.3.1 Receptors

The first step in the picornavirus infection cycle is the interaction between the virus capsid and the host cell receptor on the cell surface. The receptor-specificity is defined by the arrangement of the capsid proteins within the virion: the surfaces of many viruses in the *Enterovirus* genus are marked by deep depressions, canyons, surrounding the fivefold axis of symmetry, which often serve as an attachment site for the cellular receptor (Plevka et al., 2012, Olson et al., 1993, Colonno et al., 1988) while in contrast, foot-and-mouth disease virus (FMDV; genus *Aphthovirus*) does not possess the canyons but instead a flexible loop exposed on the virus surface serves as an attachment site for the receptor (Acharya et al., 1989). The role of receptors is not only to concentrate the viral particles to the cell surface but also to promote the infection by inducing the capsid disassembly or by targeting the particle to a specific subcellular compartment where the disassembly and genome release can occur. In addition, receptor binding may induce intracellular signaling, which mediates virus uptake into the cell. One receptor does not necessarily mediate all these functions,

thus, several picornaviruses have been found to use more than one receptor in cellular infection and according to recent findings the use of multiple receptors is more a rule than an exception (Ehrenfeld et al., 2010). Picornaviruses can utilize a wide variety of different receptor types (Tuthill et al., 2010) among which poliovirus receptor PVR (Mendelsohn et al., 1988), rhinovirus receptors ICAM-1 and LDLR (Hofer et al., 1994, Greve et al., 1989), coxsackievirus B receptors CAR and DAF (Coyne and Bergelson, 2005, Shafren et al., 1995) and integrins used by many enteroviruses and parechovirus 1 (Joki-Korpela et al., 2001, Nelsen-Saltz et al., 1999, Roivainen et al., 1994) are probably the best-studied receptors. In addition, other receptors include heparan sulfate, sialic acid and recently identified enterovirus 71 receptors SCARB2 and PSGL-1 (Tuthill et al., 2010, Nishimura et al., 2009, Yamayoshi et al., 2009).

Poliovirus receptor (PVR or CD155), coxsackie- and adenovirus receptor (CAR) and intercellular adhesion molecule-1 (ICAM-1) belong to the immunoglobulin superfamily. These receptors are glycoproteins that consist of two (CAR; Cohen et al., 2001), three (PVR; Zhang et al., 2008), or five (ICAM-1; Casasnovas et al., 1998) extracellular immunoglobulin-like (Ig-like) domains, a transmembrane domain and a cytoplasmic domain. The first Ig-like domain has been shown to bind the virus by inserting into the canyon, which triggers the penetration of the virus into the cell. PVR is the only receptor that has been shown to bind polioviruses and it has been suggested to possess an important role in the early steps of infection such as in binding of the virus to the cell surface, in the penetration to the cell and in uncoating (Ohka et al., 2004). However, its biological role in cellular functions is not fully understood. ICAM-1 allows cells to adhere to extracellular matrix or to other cells. It plays a crucial role, for example, in the migration of leukocytes from the blood to inflammation sites (van de Stolpe and van der Saag, 1996). As an unwanted property, ICAM-1 serves as a receptor for the major group of rhinoviruses (Greve et al., 1989, Staunton et al., 1989) and coxsackievirus A21 that displays dual-receptor specificity (ICAM-1 and DAF; Shafren et al., 1997) whereas the minor group of rhinoviruses (Hofer et al., 1994) utilizes members of the LDLR receptor family (low density lipoprotein receptor) as their receptors. All coxsackie B viruses (CVBs) bind to CAR that functions in cell-cell adhesion and as a barrier for the paracellular flow of solutes and macromolecules within the tight junctions in polarized epithelial cells (Cohen et al., 2001, Bergelson et al., 1997). In addition to CAR, decay accelerating factor DAF (CD55) serves as a receptor for several coxsackie B viruses and other enteroviruses (Karnauchow et al., 1996, Bergelson et al., 1994, Crowell et al., 1986). DAF is a member of the complement control family of receptors consisting of four extracellular consensus repeat modules attached to the membrane by a glycosylphosphatidyl inositol (GPI) anchor. It has been thought to function as a primary receptor for viruses concentrating the virus particles onto cell surface after which other receptors, for example CAR, are required to mediate the infectious entry (Coyne and Bergelson, 2006).

Integrins are essential cell-surface adhesion molecules that interact with other cells and with extracellular matrix proteins (Akiyama, 1996). They can mediate bidirectional signals across the plasma membrane, which helps the cell to respond rapidly to surrounding

environmental changes. Hence, they participate in many biological functions, such as cell migration, proliferation and differentiation and survival. Integrins are large, heterodimeric proteins that consist of two subunits called α and β. Both subunits are glycoproteins consisting of a large extracellular domain, a transmembrane helix and a short cytoplasmic domain (Hynes, 2002). In vertebrates, eighteen α and eight β subunits in different combinations form an integrin family with 24 members (**Figure 4**). Although some subunits appear only in a single heterodimer, 12 integrins contain the β1 subunit, and five contain αV. All five αV-, two β1-integrins (α5β1 and α8β1) and αIIbβ3 recognize arginine-glycine-asparagine (RGD) tripeptide that is present in many integrin ligands, for example in fibronectin and vitronectin (Humphries et al., 2006, Dickinson et al., 1994, Ruoslahti and Pierschbacher, 1987), and also in a few picornaviral capsids (Merilahti et al., 2012).

Two human enteroviruses, coxsackievirus A9 (CV-A9; Williams et al., 2004, Roivainen et al., 1994) and echovirus 9 (E-9; Nelsen-Saltz et al., 1999), one human parechovirus, human parechovirus 1 (HPeV-1; Seitsonen et al., 2010, Joki-Korpela et al., 2001, Pulli et al., 1997), and one animal virus, foot-and-mouth disease virus (FMDV; genus *Aphthovirus*; Duque et al., 2004, Jackson et al., 2004, 2002, and 2000), have been shown to bind to integrin receptors via RGD-motif. In human picornaviruses, RGD-motif resides near the C-terminus of VP1, which is an exposed, flexible site on the viral capsid while in FMDV RGD is located on the G-H loop of VP1 (Dicara et al., 2008, Hendry et al., 1999). CV-A9 has been shown to bind *in vitro* to αVβ3 and αVβ6 while E-9 binds to αVβ3. Similarly to CV-A9, HPeV-1 binds *in vitro* to αVβ3 and αVβ6 and, in addition, affinity to integrin αVβ1 has also been described (Pulli et al., 1997). FMDV has been shown to attach to several αV-integrins *in vitro* but it has been suggested that integrin αVβ6 serves as a true virus receptor in natural infections (Monaghan et al., 2005). However, the integrin-virus interaction has not been shown to trigger viral capsid alterations that are essential for the infectious entry, hence other (co)receptors are necessary for completing the virus internalization (Tuthill et al., 2010).

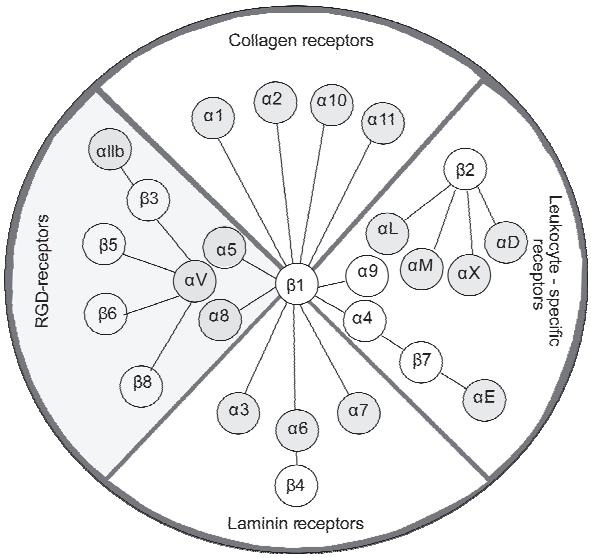


**Figure 4**. Integrin family consists of 24 members that are divided into four groups based on their ligands. RGD-binding integrins include five αV-integrins (αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8), two β1 integrins (α5β1 and α8β1) and αIIbβ3. (Modified from Barczyk et al., 2010).

### 2.3.2 Particle uncoating and genome release

After the picornavirus has bound to its receptor, it penetrates through the plasma membrane to release the RNA genome or moves towards the cell interior within an endosomal vesicle where the particle uncoating and RNA release occur. The mechanisms of particle penetration and uncoating are poorly defined and have been studied only with relatively few picornavirus types such as poliovirus 1, rhinovirus 2 and 14, and FMDV. In many enteroviruses, including polio- and major group rhinoviruses, binding to receptors induces drastic changes in the particle conformation that result in the externalization of VP4 and the N-terminus of VP1 leading to the formation of an A (altered)-particle (135S; Hewat and Blaas, 2004, Greve et al., 1991, Fricks and Hogle, 1990). VP4 and the N-terminus of VP1 have been shown to be inserted into membranes (Tuthill et al., 2006, Danthi et al., 2003, Fricks and Hogle, 1990) allowing the virus particle to anchor to the cellular plasma or vesicle membrane and presumably to form channels and pores through which the RNA genome can be transported into the cytosol (Brandenburg et al., 2007, Tosteson et al., 2004, Danthi et al., 2003). When the infection proceeds, the level of membrane-associated A-particles decreases and the second form of the altered particle (80S) appears. 80S lacks the RNA and is called "empty capsid" (Fricks and Hogle, 1990). Although the receptor binding of minor group rhinoviruses does not trigger the alteration in the virus capsid, the stages of uncoating are similar to the major group rhinoviruses (reviewed by Fuchs and Blaas, 2010). While poliovirus uncoating occurs independently of low pH (Wetz and Kucinski, 1991), the uncoating of rhinoviruses is pH dependent (Giranda et al., 1992) implying that polio- and rhinoviruses reside in a different cellular compartment during virus infection and particle uncoating. In contrast to the uncoating of enterovirus particles during which the icosahedral structure is maintained, it has been suggested that acid labile aphtho- and cardioviruses release their VP4 and RNA by dissociation into pentameric subunits (Dubra et al., 1982, Mak et al., 1970). However, Tuthill et al. (2009) suggested that aphthoviruses maintain the icosahedral integrity until the genome is safely transported into the cytoplasm where the particle dissociation and genome release occur. The ability to maintain the capsid structure during the viral entry thus protects the viral genome and could be a common mechanism for all picornaviruses to ensure successful RNA release and virus proliferation.

### 2.3.3 Protein processing

The picornaviral genome is translated into a single, approximately 2200 amino acid residues long polyprotein where the structural proteins VP1-4 are located within the N-terminal one third of the polyprotein (P1) and the remainder of the polyprotein includes non-structural proteins (P2 and P3; **Figure 2A and Figure 3**). The polyprotein is cleaved by viral-encoded proteases into three precursor proteins, P1, P2 and P3, which are further processed into smaller polypeptides. P1 precursor protein is modified by the covalent addition of a myristic acid residue in the majority of picornaviruses and the residue is thought to participate in the particle assembly and virus entry (Ehrenfeld et al.,

2010). P1 is cleaved into viral capsid proteins VP1, VP3 and VP0 that is further processed into VP4 and VP2 in most picornaviruses. Despite the proteolytic cleavage, VP1, VP3 and VP0 do not physically separate; they stay together as a protomer unit throughout the particle morphogenesis (Arnold et al., 1987, Grigera et al., 1985, Palmenberg, 1982). The precursor proteins P2 and P3 include the non-structural proteins that participate in the viral RNA translation and replication and interfere with cellular processes that could restrict virus replication (Teterina et al., 2011). P2 polypeptide is cleaved into 2A, precursor 2BC, 2B and 2C proteins and P3 polypeptide first into the stable precursor polypeptides 3AB and 3CD, and further into 3A, 3B, 3C and 3D proteins (**Figure 3**, step 4).

The sequence and function of 2A proteins derived from the P2 precursor vary widely in different virus species (Hughes and Stanway, 2000). For enteroviruses, for example, 2A serves as a proteinase that catalyzes the cleavages of host cell proteins and the viral polyprotein in its own N terminus yielding P1 and P2P3 precursors (Sommergruber et al., 1989, Kräusslich et al., 1987, Toyoda et al., 1986), while in aphtho-, cardio-, and kobuviruses, 2A does not possess enzymatic activity (Sasaki and Taniguchi, 2008, Donnelly et al., 2001). 2B and 2C including their stable precursor 2BC have been implicated in the production of the membranous vesicular structures where the viral replication takes place (Cho et al., 1994). In addition, 2B protein of enteroviruses has also been found localized in the endoplasmic reticulum (ER) and Golgi apparatus where it lowers the $Ca^{2+}$ levels (Ito et al., 2012, de Jong et al., 2003, van Kuppeveld et al., 1997, Sandoval and Carrasco 1997) preventing the infected cell from entering apoptosis (van Kuppeveld et al., 2005). 2C protein possesses ATPase and RNA-binding activities (Sweeney et al., 2010, Rodriguez and Carrasco, 1993).

The viral proteins derived from the P3 precursor are directly involved in RNA synthesis. Precursor proteins 3AB and 3CD participate in the replication functions as well as the smaller proteins 3A, 3B, 3C and 3D. 3AB, for example, plays multiple roles in viral RNA replication complex formation and functions and in viral RNA synthesis (Towner et al., 1996). Proteolytic cleavage of 3AB into 3A and 3B is mediated by the main viral proteases 3C/3CD, which also complete the cleavage of the precursor protein P2P3 into P2 and P3. Because of the essential role of 3C in viral maturation, this protein is the most promising target for antiviral drug design (Norder et al., 2011, De Palma et al., 2008). In polio- and coxsackievirus B3, the derived 3A protein has been shown to inhibit the membrane and secretory protein traffic from ER to Golgi and induce specific translocation of ADP-ribosylation factor (Arf) family members to membranes (Choe et al., 2005, Belov et al., 2005, Doedens et al., 1997, Lama et al., 1994). 3B (VPg) is a small, 20-24 amino acid residues long, uridylylated peptide that resides in the 5'terminus of the viral RNA serving as a primer for the initiation of the viral RNA synthesis (Paul et al., 1998). 3D serves as an RNA-dependent RNA polymerase ($3D^{pol}$) that is vital for virus replication since mammalian cells do not possess the activity by which the RNA is replicated into RNA. $3D^{pol}$ has been shown to catalyze the uridylylation of VPg into

VPg-pUpU (Pathak et al., 2008) and during the infection, 3D and VPg-pUpU with other essential elements localize to the viral replication complex where the synthesis of plus- and minus-strand genome RNAs occur (Lyle et al., 2002).

### 2.3.4 Genome replication, particle assembly and release

After polyprotein processing, the genomic RNA is synthesized in the replication complex located on the outside surface of replication vesicles that are distributed throughout the cytoplasm (**Figure 3**). Replication complex includes the viral RNA, viral-coded and host-coded proteins and host membranes, which are brought together by numerous interactions. Although the mechanisms of RNA replication are not fully understood, it is generally thought that neither actively translated RNAs nor the RNAs that have not yet been translated can serve as templates for RNA synthesis (Gamarnik and Andino, 1998, Novak and Kirkegaard, 1994). However, according to Murray et al. (2004), the replication of poliovirus RNA does not directly depend on template RNA translation. During RNA replication, the positive-strand RNA is copied into a complementary negative-strand RNA, which is then copied again into numerous positive-strand RNA progeny (Wimmer et al., 1993, Novak and Kirkegaard, 1991). Virus-encoded $3D^{pol}$ catalyzes the synthesis of both strands with the other viral and cellular factors. $3D^{pol}$ also mediates the uridylation of the VPg that serves as a primer for RNA replication and is covalently linked to the 5'end of each negative- and positive-strand RNA molecule (Pettersson et al., 1978). In addition, the 5'cloverleaf structure of enteroviruses interacts with viral and cellular proteins forming a ribonucleoprotein (RNP) complex that is essential for initiating the synthesis of new plus strands (Herold and Andino, 2000, Andino et al., 1993).

While the RNA genome is replicating in the replication complex, the empty capsids for new viral particles are formed from the structural proteins VP0, VP1 and VP3 (**Figure 3**, step 7). First, they assemble into pentameric subunits and then into icosahedral "procapsids". Newly synthesized plus-strand RNA is then packed into the "procapsid" by the mechanism that is not well understood: the genome may be inserted into the procapsid or pentamers may condense around the RNA that is emerging from the replication complex (Pfister et al., 1995, Jacobson and Baltimore, 1968). However, Liu et al. (2010) have proposed a model for enteroviral encapsidation where $2C^{ATPase}$ that is associated with replication complex at the membrane binds directly to the pentamer via VP3 after which the pentamer binds VPg-linked genomic RNA (Nugent and Kirkegaard, 1995). This model suggests that assembly and encapsidation occur in a close contact with the membranous environment and is dependent on protein-protein interactions within the active replication complex. At the final step, enteroviral VP0 is cleaved to VP4 and VP2 by a mechanism that involves an RNA-dependent autocatalytic process (Racaniello, 2007). This leads the provirions (capsid and RNA) to mature into virions that are eventually released by cell lysis. However, the final steps differ in picornaviruses since, for example, human parechoviruses do not possess the maturation cleavage of VP0 (Stanway et al., 2000). Thus the mechanism by which parechoviruses assemble into virions remains to be studied.

## 2.4 Endocytosis

After binding to cell surface receptors, viruses have to enter cells to initiate productive infection. Since viruses are simple in structure and lack the capacity to proliferate on their own, they hijack the cellular transport systems. Endocytosis is a general mechanism of the host cell to transport cargo molecules such as membrane components, fluid and solutes into the cytoplasm and out from the cell (Ewers and Helenius, 2011, Pelkmans and Helenius, 2003). Although some of the viruses enter cells directly through the plasma membrane, most viruses prefer endocytosis because it offers many benefits, for example, a delay in immune response, built-in transport mechanism across the plasma membrane and access to intracellular organelles where viruses can safely uncoat and release their genome (Schelhaas, 2010). Previously, it was thought that receptor-mediated endocytosis generally leads to clathrin-mediated endocytosis (CME) but currently it has become apparent that macromolecular endocytosis is a much more diverse and complicated process. The best-characterized endocytosis pathways are the clathrin-mediated route, caveolin/raft-dependent entry and macropinocytosis, but several other pathways have also been identified (**Figure 5**). In addition, the use of the same receptor does not necessarily lead to the same entry pathway (Kim and Bergelson, 2012), and the same virus species or type may use multiple receptors for endocytosis in a cell line-dependent manner.

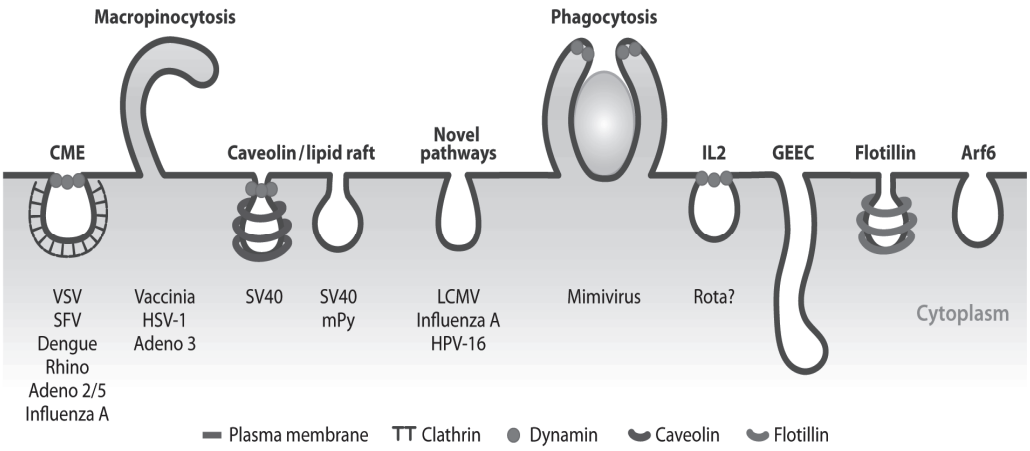


**Figure 5**. Endocytic pathways of the cell. Endocytosis is a general way for cells to carry substances into the cell interior and back to the surrounding environment. Viruses have evolved to utilize these mechanisms that give them a free ride into the cell. The names of the known endocytic pathways are shown above the plasma membrane and examples of viruses known to utilize the named routes are listed beneath the membrane (Modified from Mercer et al., 2010).

Endocytic pathways differ in the size of the endocytic vesicles, the nature of the cargo and the mechanism of vesicle formation (Schelhaas, 2010, Ghigo, 2010). In addition, the formation of primary endocytic vesicles (PEVs) involves a large number of cellular factors, such as coat proteins, adaptors, scission and regulatory factors

and molecules that promote trafficking which differ in the various pathways and are only partially known (Mercer et al., 2010). However, the overall steps are similar (**Figure 6**): endocytosis starts with the formation of primary endocytic vesicles that are first pinched off the plasma membrane and then targeted to endosomes that are, for example, responsible for sorting, recycling and degradation of cargo molecules/ viruses. The main endosomal organelles can be divided into early endosomes (EE), maturing endosomes (ME), recycling endosomes (RE), late endosomes (LE) and lysosomes (Mercer et al., 2010, Schelhaas, 2010). Different Rab GTPases are used to specify the organelles: Rab5 is located in EE, Rab4 is associated with fast recycling and Rab11/22 with slow recycling endosomes. Both Rab5 and Rab7 also exist in ME and Rab7 and Rab9 reside in LE (Schelhaas, 2010). EEs reside usually on the periphery of the cell and are the main sorting stations for cargo. They are complex organelles with several different tubular and vacuolar domains; tubular elements are involved in the transport of recycling cargo and vacuolar domains dissociate and mature into LEs through intermediate MEs that contain markers of both early and late endosomes (Grant and Donaldson, 2009, Rink et al., 2005, Marsh et al., 1986). Lakadamyali et al. (2006) have shown that there are at least two variants of EE that differ in the rate by which they convert to LE. LEs then fuse with lysosomes where active degradation takes place. Viruses are too big to utilize narrow tubular extensions and are thus generally localized in vacuolar domains that are sorted to the degradative pathway (Mercer et al., 2010).

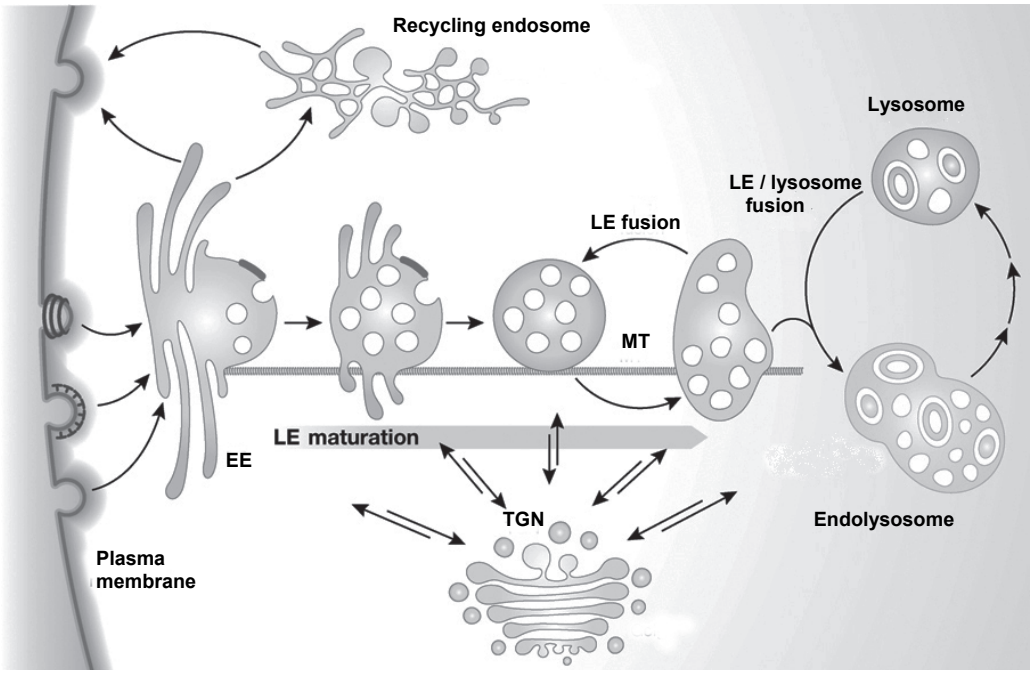


**Figure 6.** Endosome and lysosome maturation. EE=early endosome, LE=late endosome, MT=microtubules and TGN=trans Golgi network (Modified from Huotari and Helenius, 2011).

Modern techniques such as imaging of virus entry in living cells, modern light microscopy analysis (e.g. confocal, FLIM and STED) with specific endocytic markers and inhibition of specific endocytic routes by siRNAs, dominant-negative constructs and chemical inhibitors have helped to enlighten detailed mechanisms of virus endocytosis (Kim and Bergelson, 2012, Bergelson, 2008, Ewers et al., 2005, Brauchle et al., 2002). However, the information that is now available rather decreases than increases the understanding of virus endocytosis: only few endocytic mechanisms were previously thought to be utilized by viruses whereas now the complexities of the cellular uptake and vesicular transport network have become apparent. In addition, endocytic routes were previously suggested to occur separately whereas now the cross-talk between the separate routes has been discovered. Picornaviruses are good tools for endocytosis studies since most members of the family grow in cell cultures, many of those are well-characterized, several cellular receptors have been identified, they are amenable to structural studies and several important intermediates in the cell entry route have been identified (Ehrenfeld et al., 2010). The following chapters will describe the characteristics of the major endocytic routes utilized by some picornaviruses.

### 2.4.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the best-characterized, rapid and efficient endocytic route that occurs continuously in all mammalian cells. A characteristic feature of CME is the clustering of ligated transmembrane receptors into clathrin-coated vesicles (CCV) of about 120 nm in size (Chigo, 2010). CME is involved in the uptake of nutrients essential for cells such as growth factors, antigens and recycling receptors (Takei and Haucke, 2001). The formation of CCV occurs in four steps starting with the assembly of the clathrin-coated pit and bud formation on the cytoplasmic site of the plasma membrane (**Figure 5**). In the next step, clathrin-coated pit matures and the forming vesicle is released from the plasma membrane by the action of large GTPases, dynamins (Hinshaw, 2000, Damke et al., 1994). In the final stage, CCV uncoats and the contents are transported to early endosomes (Doherty and McMahon, 2009, Conner and Schmid, 2003, Takei and Haucke, 2001). Traditionally it was thought that the initiation of clathrin-coated pit is dependent on the highly conserved protein AP2 but recent studies using yeast and mammalian cells indicate that a set of proteins form a module that defines the specific sites on the plasma membrane where clathrins are transported from the surrounding regions (Suzuki et al., 2012, Henne et al., 2010, Stimpson et al., 2009). The module includes FCH domain only (FCHO) proteins, EGFR pathway substrate 15 (Eps15) and intersectins in addition to the plasma membrane specific lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$), which indicates that the module formation occurs only on the plasma membrane. Module proteins recruit adaptor proteins (AP2) that in turn recruit clathrin and AP180 including other adaptors involved in cargo selection (Henne et al., 2010, Ungewickell and Hinrichsen, 2007). However, AP2 is not always required for CME (Motley et al., 2003) implicating that the composition of clathrin-coated pits is not always identical. Cargo usually reaches the EE in less than 2 min after internalization,

is seen in LE after 10-12 min, and in lysosomes within 30-60 min (Lakadamyali et al., 2006, Mukherjee and Maxfield, 2004, Kielian et al., 1986).

Several viruses have been shown to induce the formation of clathrin-coated pits including members of the picornavirus family. From clathrin-coated vesicles viruses are usually transported to early endosome where they encounter acidic pH, which is necessary for uncoating of many viruses. For example FMDV and rhinoviruses have been shown to depend on acidic compartments since the blocking of acidification inhibits virus infection (Johns et al., 2009, Prchla et al., 1994, Baxt 1987). Although O'Donnell et al. (2008) have shown that FMDV can be forced to enter the cell via an alternative caveolin-dependent pathway it still retains the requirement for acidic pH, which indicates that the cellular endocytic pathways cross-talk with each other. In addition, a recent study by Lin et al. (2012) suggests that enterovirus 71 utilizes the clathrin-mediated and pH-dependent pathway for entering the cell. However, most enteroviruses survive the low pH in gastrointestinal tract, since they are transmitted via fecal-oral route and hence their capsids must be stable at low pH (Kim and Bergelson, 2012).

### 2.4.2 Caveolin-dependent endocytosis

Caveolin-mediated endocytosis occurs via caveolae (Yamada, 1955, Palade, 1953). This route is dependent on cholesterol, lipid rafts and complex signaling pathways involving tyrosine kinases and phosphatases (Mercer et al., 2010). Caveolae internalize membrane components such as glycosphingolipids and glycosylphosphatidylinositol-anchored proteins, special extracellular ligands and bacterial toxins, but the uptake and forward movement are slow and asynchronous. Unlike clathrin-mediated endocytosis, internalization through caveolae is a ligand triggered event. Caveolae are flask-shaped invaginations, typically of 60-80 nm in diameter (**Figure 5**) and they are present on the plasma membrane of many cell types (Parton and Simons, 2007, Yamada, 1955, Palade, 1953). Although caveolae do not have a characteristic electron-dense layer on their cytosolic surface in contrast to clathrin-coated vesicles, their "coat" is primarily composed of caveolin-1 and caveolin-2 proteins in non-muscle cells or caveolin-3 in muscle cells (Hayer et al., 2010, Tang et al., 1996, Rothberg et al., 1992). Caveolae also contain cavins (Bastiani et al., 2009, McMahon et al., 2009) and dynamins that reside in the neck of the flask-shaped caveolar indentation and are probably involved in pinching off the caveolar vesicles from the plasma membrane (Oh et al., 1998). Although it was previously suggested that caveolar vesicles fuse either with early endosomes or with each other forming novel vesicles called caveosomes, which do not include early endosome antigen 1 (EEA1) and have neutral pH in contrast to CCVs and EEs, Hayer et al. (2010) have recently demonstrated that the "caveosomes" actually correspond to modified late endosomes or lysosomes. These results suggest that the caveolae are part of the classical endocytic pathway, which refutes the earlier model of caveolae disassembly and caveosome formation (Pelkmans et al., 2004 and 2001). Thus, the discoverers of "caveosomes" suggest the term not to be used any longer (Hayer et al. 2010).

Echovirus 1 (E-1) and coxsackievirus B3 (CV-B3) are the most studied picornaviruses shown to utilize a pathway that has the characteristic features of caveolin-mediated entry. In addition, FMDV has been shown to use caveolin-mediated pathway when the binding to the cell surface occurs via heparan sulfate instead of αV-integrins (O'Donnell et al. 2008). Pietiäinen et al. (2004) have shown by using video-enhanced live cell microscopy, biochemical characterization and inhibitors that echovirus 1 binds to its integrin receptor α2β1, is localized to lipid microdomains (rafts) and is imported into caveosomes or modified late endosomes (Hayer et al. 2010), where the virus colocalizes with caveolin and α2β1. However, further studies have indicated that the entry of E-1 occurs via macropinocytosis (Karjalainen et al., 2008, Liberali et al., 2008) and that the caveosomes are the final target for E-1. Furthermore, Karjalainen et al. (2011) have shown that E-1 accumulates in multivesicular bodies, which are distinct from acidic late endosomes or lysosomes. Following this, Rintanen et al. (2012) have identified a novel, nonrecycling, calpain-dependent degradative route for E-1. Interestingly, when two different strains of CV-B3 were studied, RD strain was shown to utilize the caveolin-1 mediated endocytic pathway while H3 strain was dependent on clathrin-mediated and pH-dependent internalization (Coyne et al., 2007, Chung et al., 2005). Similarly to E-1, the early stages of entry of the RD strain also had features of macropinocytosis (Karjalainen et al., 2008, Liberali et al., 2008, Coyne et al., 2007). The different results between RD and H3 strains may, however, be due to different cell types used in the studies: endocytosis of H3 strain was followed in non-polarized HeLa cells, whereas RD strain was studied in polarized CaCo2 cells. Thus, it is evident that some picornaviruses utilize different entry pathways in different cell lines.

### 2.4.3 Macropinocytosis and phagocytosis

Macropinocytosis and phagocytosis require extensive actin cytoskeletal reorganization, which leads to the outward-directed formation of plasma membrane extensions in contrast to other pathways where the plasma membrane buds into the cell (**Figure 5**). Phagocytosis is not, however, commonly used for viral entry but rather the uptake mechanism for bacterial or other large particles. Instead, macropinocytosis has emerged as the major endocytic mechanism being used by more than 20 different animal viruses (Mercer and Helenius, 2012). Macropinocytosis is not a continuously ongoing process in most cell types and active macropinocytosis operates only for a limited time. The pathway is responsible for nonspecific uptake of fluid, solutes, membrane, ligands and smaller particles that are attached to the plasma membrane. The pathway can be induced by growth factors, which enhance the generation of macropinosomes through membrane ruffles that can take the form of filopodia or blebs. Resulting macropinosomes vary in shape and size and they are relative large with diameters from 0.2 μm up to 10 μm (Schelhaas, 2010, Swanson et al., 2008). The activation and formation of macropinosomes involve numerous factors that vary in different cell lines and are somewhat dependent on internalized cargo. Activation of macropinosomes and PEVs requires, for example, sodium/proton exchanger, various cellular kinases, the RhoGTPases Rac1 and Cdc42,

cholesterol and GTPases such as Rab34 and Arf6 (Mercer et al., 2010, Schelhaas, 2010, Mercer and Helenius, 2009). Formed macropinosomes detach from the plasma membrane and move deeper into the cytoplasm where they are sensitive to the cytoplasmic pH, undergo acidification and homo- and heterotypic fusions or they can be recycled back to the plasma membrane (Hewlett et al., 1994, West et al., 1989). Maturation involves the gain and loss of early and late endosomal markers before fusion with lysosomes (Racoosin and Swanson, 1993) showing that some mechanisms that are involved in macropinocytosis are shared with other endocytic pathways.

According to Mercer and Helenius (2012), the nonspecific nature of macropinocytosis is favorable for viruses since uptake through this pathway is not dependent on specific receptors and therefore viruses can initially bind to any surface component, which then triggers the appropriate receptors to turn on the macropinocytosis pathway. Picornaviruses that are reported to use macropinocytosis as their cellular entry include E-1 and coxsackie B viruses. They share a similar entry pathway where the first steps are characteristics of macropinocytosis and the rest of the entry shares the features of other endocytic routes (Karjalainen et al., 2008, Coyne et al., 2007). In addition to E-1 and CV-B3, rhinoviruses 8 and 14 have been shown to utilize macropinocytosis (Khan et al., 2011 and 2010). However, some features of rhinovirus 8 endocytosis contradict the criteria for macropinocytosis (Mercer and Helenius, 2012 and 2009) and, interestingly, seem to be similar to the endocytic features of coxsackievirus A9, which is the topic of this thesis.

### 2.4.4 Non-clathrin- and non-caveolin-mediated endocytosis

The endocytic pathways including IL2 (interleukin 2), GEEC, Flotillin-dependent, Arf6-mediated and novel pathways presented in **Figure 5** are representatives of non-clathrin- and non-caveolin-mediated endocytic routes. What is common to all of them is the lack of detectable coats, and independence of clathrin and caveolin. Some of them have been shown to transport viruses to the endosomal networks although the pathways are still poorly characterized. For example, the uptake of rotaviruses (family *Reovirus*) has been shown to be dependent on dynamin-2 and cholesterol suggesting a possible relationship with the uptake of IL2 (Sauvonnet et al., 2005, López and Arias, 2004, Lamaze et al., 2001). Flotillins are membrane-bound proteins that share a similar membrane topology with caveolins. Flotillin 1 and flotillin 2 form microdomains at the plasma membrane after which the formed flotillin-positive PEVs fuse with EEs (Babuke et al., 2009). This pathway has been implicated in the internalization of GPI-anchored proteins and proteoglycans but has not yet been identified as an endocytic route for any virus (Mercer et al., 2010, Hansen and Nichols, 2009, Payne et al., 2007). Another pathway that mediates the internalization of the GPI-anchored proteins is GEEC (GPI-anchored enriched endocytic compartments; Sabharanjak et al., 2002). Endocytosis in this pathway is dependent on lipid microdomains but not dynamin and has not yet been observed to transport any viruses.

GPI-linked protein CD59 and major histocompatibility complex (MHC) class I proteins can be cointernalized into Arf6-positive endosomes that are distinct from GEEC endocytic structures (Lundmark et al., 2008, Naslavsky et al., 2004). Internalization is sensitive to cholesterol depletion and includes membrane ruffling initiated by Arf6 (Naslavsky et al., 2004 and 2003). After PEV formation, Arf6-positive endosome fuses with early endosome or ends up to the Arf6-dependent recycling pathway (Naslavsky et al., 2004). However, Arf6 is also implicated in macropinocytosis and clathrin-mediated endocytosis, and it has been shown to have a role in the endocytosis of baculoviruses and human immunodeficiency virus type 1 (HIV-1; García-Expósito et al., 2011, Laakkonen et al., 2009). In this thesis it is demonstrated that Arf6 is essential for CV-A9 endocytosis in at least two cell lines and that the virus is endocytosed in Arf6-positive endocytic vesicles (II and III).

## 2.5 Picornaviruses as work horses in research and medicine

Although viruses are generally thought to be unwanted visitors, they also serve as helpful and important tools in research and medicine. As described above, picornaviruses are good tools for studying the cellular uptake and endocytosis mechanisms but they (especially enteroviruses) are also potential viral vectors for vaccines and gene therapy (Henke et al., 2008, Chapman et al., 2000, Andino et al., 1994). In addition, the use of picornaviruses as vectors in cancer therapy has recently been demonstrated (Au et al., 2011 and 2007, Berry et al., 2008, Shafren et al., 2004). The small genome size enables the modification of the genome without laborious subcloning steps but also limits the stability of the modified genomes with large inserts. Since picornaviruses are prone to recombination, this may lead to the removal of an insert during virus proliferation. In addition, the location of the insert must be designed so that it does not interfere with the genome translation. Nevertheless, the abilities of picornaviruses to penetrate specific target cells and to replicate there make them valuable tools as gene transfer vehicles to deliver foreign genes into living organisms.

Information gathered from studies with polioviruses and coxsackieviruses make them potential choices as gene transfer vehicles. Particularly coxsackievirus B3 (CV-B3) has been important in method development. So far, extra genetic material has successfully been inserted into four sites within the picornaviral genome. An insert can be placed into the open reading frame of VP1 but its size is greatly restricted because of conformational constraints during virus assembly (Slifka et al., 2001, Reimann et al., 1991, Burke et al., 1988). Internal ribosome entry site (IRES) of another enterovirus was used when the foreign gene was located in the 5' end of the native IRES. This method requires a short foreign insert for stable expression (Slifka et al., 2001, Alexander et al., 1994). VP1 gene can also be replaced with a foreign gene but this method results in defective, noninfectious viral genome, which is encapsidated only in the cell lines that provide the missing VP1. For example, Meyer et al. (2004) successfully inserted the GFP-luciferase reporter gene to the P1 region but this recombinant CV-B3 did not produce progeny viruses in cultured cells. The most successful way of making stable insertions is to place

an insert immediately downstream of the start codon in the VP4 gene or at the junction between the VP1 and 2A genes together with artificial cleavage sites recognized by viral proteases 2A or 3C. This strategy leads to the release of the foreign peptide or protein from the viral proteins by the action of viral proteases and the size of the foreign gene can be up to 700-750 nucleotides. Most recombinant CV-B3s, as well as the GFP-expressing coxsackievirus A9 designed in this thesis have been created in this manner (Heikkilä et al., 2011 (IV), Kim et al., 2010, Miller et al., 2009, Lim et al., 2005, Slifka et al., 2001, Henke et al., 2001, Chapman et al., 2000).

Picornaviruses can be used in cancer and gene therapy either in native, non-modified form, or after genetic modification. Chapman et al. (2000) demonstrated that an attenuated recombinant coxsackievirus vector that produces a fully bioactive cytokine can be generated: they placed the murine interleukin-4 coding sequence between the capsid coding region in P1 and the 2A protease coding region in P2 and the insert was expressed during viral replication implying that it is possible to manipulate the immune response. In addition, Henke et al. (2001) have generated several recombinant cytokine producing CV-B3 variants in order to study whether the cytokine expression is able to protect against a viral disease or to decrease the clinical symptoms during an ongoing infection. In their study gamma interferon (IFN-γ) seemed to be a potential protector against CV-B3 replication *in vivo*. Furthermore, Henke et al. (2008) have shown that the local and simultaneous expression of IFN-γ by the virus itself led to a strong and long-term activation of the immune system and protection against lethal infection in mice. Kim et al. (2011), instead, injected mice with recombinant CV-B3 expressing fibroblast growth factor 2 (FGF2), which significantly improved the blood flow in ischemic limbs for over three weeks supporting its use as a novel viral vector for gene therapy. The virulence of this recombinant virus was also drastically attenuated. In addition, Dan and Chantler (2011) designed a coxsackievirus vector that delivers the glucagon-like peptide 1 (GLP-1) specifically to the pancreas making the vector a possible therapeutic tool for pancreas-related diseases such as type 1 and 2 diabetes, pancreatitis and pancreatic cancer.

The potential of picornavirus types echovirus 1, 12, 17 and 26 and coxsackievirus A21, A13, A15 and A18 in cancer therapy has only recently been realized (Israelsson et al., 2011, Au et al., 2011 and 2007, Berry et al., 2008, Shafren et al., 2004). However, in these studies the picornaviruses have not been genetically modified, and the safety aspect relies on their low pathogenesis in humans. In addition to human picornaviruses, animal picornaviruses such as Seneca Valley virus 001 (SVV-001) have been of interest because they are asymptomatic in humans but still capable of destroying human cancer cells (Reddy et al., 2007). The results with SVV-001 have also been promising in clinical trials (Rudin et al., 2011) and are approaching phase II studies with small cell lung cancer and pediatric neuroendocrine cancer patients. Although picornaviruses possess many benefits in cancer therapy and gene delivery, including non-integration into host DNA, absence of viral oncogenes that may lead to tumorgenesis, ease of genetic manipulation by reverse genetics systems and mainly asymptomatic nature of infection, there are also

obstacles to overcome. Because enteroviruses are widespread, most human populations contain neutralizing antibodies, which may be a problem if enteroviruses are used as a gene deliver vectors. Even though Henke et al. (2008) and Kim et al. (2009) have shown that recombinant CV-B3 can produce foreign proteins despite the presence of neutralizing antibodies, the attenuated strains of CV-B3 are subject to relatively rapid elimination by the host leading to instability of foreign gene expression (Kim et al., 2010). The inherent high error rates of RNA-dependent RNA polymerases that result in genetic variability (Domingo and Holland, 1992) and the possibility of persistent infections that may cause chronic diseases are grave concerns for clinical applications using picornaviruses (Kim and Nam, 2011).

## 2.6 Coxsackievirus A9

Coxsackievirus was first isolated in 1948 in Coxsackie, New York, from a young boy who was suffering from acute flaccid paralysis (Dalldorf & Sickles 1948). Later, coxsackieviruses were divided into two subgroups A and B according to the pathogenicity in newborn mice: group A viruses affected striated muscles whereas group B viruses infected central nervous system, exocrine pancreas and liver (Hyypiä et al., 1993). Although coxsackievirus A9 (CV-A9) was first classified into group A, which still defines its name, Chang et al. (1989) and Pulli et al. (1995) showed that genetically CV-A9 represents CV-B viruses rather than CV-A and therefore it now belongs to the *Enterovirus B* species in the genus *Enterovirus*.

CV-A9 is a common and widespread human pathogen that usually causes mild, cold-like symptoms. However, it can also cause serious diseases affecting the heart, central nervous system and pancreas. In addition, CV-A9 has been associated with type 1 diabetes (Roivainen et al., 1998, Hyöty et al., 1995). Recently two outbreaks of CV-A9 infection have been reported in China 2005 and in Japan 2012. In China CV-A9 was mainly responsible for the outbreak of aseptic meningitis in 85 children, all less than 13 years old and majority of 4-5 years, whereas in Japan CV-A9 caused exanthema in 50 children and infants, all less than 6 years old (Aoki et al., 2012, Cui et al., 2010) implying that CV-A9 is generally a nuisance of young children.

### 2.6.1 General properties

The complete nucleotide sequence of coxsackievirus A9 genome has been determined by Chang et al. in 1989. Comparison of CV-A9 sequence with other picornaviruses revealed significant amino acid similarity but also, and in contrast to other enteroviruses, CV-A9 was found to possess an insertion of about 15 amino acids with arginine-glycine-asparagine (RGD) tripeptide at the C-terminus of VP1. Since RGD-motif is known to be the cell attachment motif of a number of adhesive extracellular matrix, blood and cell surface proteins and it is recognized by several integrins (Ruoslahti, 1996, Ruoslahti and Pierschbacher, 1987), numerous studies have focused on investigating the role of integrins

and RGD-motif in CV-A9 binding. However, some of these results are contradictory (Williams et al., 2004, Triantafilou et al., 2000, Roivainen et al., 1996, 1994 and 1991, Hughes et al., 1995). Santti et al. (2000) showed that all 35 clinical isolates of CV-A9 collected from different geographical regions during five decades contained RGD-motif, which implies its importance in clinical CV-A9 infections. Unfortunately, RGD-motif was not visible in the crystal structure of CV-A9, which has been resolved to 2.9 Å resolution in complex with the antiviral compound WIN 51711 (**Figure 7**; Hendry et al., 1999). In contrast to most picornaviruses, CV-A9 has five distinct, about 15 Å deep depressions around each fivefold protrusion rather than a continuous circular canyon, and the VP1 C-terminal portion, which contains the flexible RGD-motif, resides on the outer surface of the capsid. Although the structure and genome of CV-A9 resemble coxsackie B viruses, the regions that define the receptor binding sites have differences indicating differential receptor recognition by CV-A9.

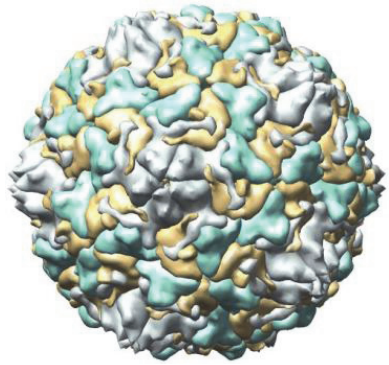

**Figure 7**. The crystal structure of coxsackievirus A9. VP1 proteins and the formed craters are shown as grey, VP2 proteins as turquoise and VP3 proteins as gold. (Hendry et al., 1999).

### 2.6.2 Receptors and endocytosis

Several biochemical studies have demonstrated that coxsackievirus A9 binds to integrins αVβ3 and αVβ6 via RGD-motif (Williams et al., 2004, Triantafilou et al., 1999, Roivainen et al., 1994). However, it has remained unclear whether integrins mediate internalization and RNA release. Recently, Shakeel et al. (2013) have used surface plasmon resonance, electron cryo-microscopy and image reconstruction to analyze the *in vitro* interactions between CV-A9 and αVβ6. They show that integrin αVβ6 binds to the viral capsid close to the predicted RGD-loop with nanomolar affinity but does not induce uncoating. This indicates that other receptors are necessary for completing virus endocytosis and genome release. In addition to integrin-mediated binding, RGD- and integrin-independent attachment and internalization of CV-A9 have been shown to exist (Triantafilou et al. 2000, Roivainen et al. 1996, Hughes et al. 1995). Furthermore, it has been suggested that MHC class I associated protein β2-microglobulin (β2M) and the endoplasmic reticulum chaperone HSPA5 (GRP78) mediate CV-A9 attachment and endocytosis (Triantafilou et al., 2002, 2001, 2000, 1999).

MHC class I (MHC-I) molecules are cell surface recognition elements that are expressed in all somatic cells. Their role is to participate in the immune system by presenting

peptides derived mainly from cytosolic proteins to T-cells. Structurally (**Figure 8**) MHC-I comprises a heavy chain (α), a light chain (β2M) and a short antigenic peptide (Natarajan et al., 1999, Bjorkman et al., 1987) and the assembly of MHC-I molecules occurs in the endoplasmic reticulum. In normal healthy cells, peptides that assemble with MHC-I are derived from the cell's own proteins while during infections, a subset of MHC-I molecules becomes associated with pathogen-derived peptides, which activates the T-cell response (Li and Raghavan, 2010). However, certain viruses such as polioviruses (Deitz et al., 2000) are capable of down-regulating the expression of MHC-I molecules, which makes cells resistant to immune response. Triantafilou et al. (1999) have shown by using blocking antibodies and flow cytometric analysis that antibodies against MHC-I and β2M prevent CV-A9 infection completely but the virus does not bind directly to β2M. This suggested that β2M functions in a postattachment stage rather than as a receptor molecule.

HSPA5 protein (GRP78), however, has been shown to participate in CV-A9 binding to GMK (green monkey kidney) cell surface together with αVβ3 integrin (Triantafilou et al., 2002 and 2000). HSPA5 protein is present in all cells and plays an important role in maintaining cellular homeostasis (Kiang and Tsokos, 1998). Although HSPA5 is best known as an ER luminal protein, it can also reside on the plasma membrane where it functions as a receptor for numerous ligands (Gonzalez-Gronow et al., 2009). The structure of transmembrane HSPA5 is suggested to comprise four hydrophobic domains that integrate the protein on the membrane and two distinct functional units: N-terminal nucleotide binding domain contains ATPase activity and C-terminal domain binds the substrate (Wisniewska et al., 2010, Conzalez-Gronow et al., 2009). The functions of HSPA5 are determined by the co-molecules MTJ-1 (DnaJ-like protein 1; Misra et al., 2005) or MHC-I (Conzalez-Gronow et al., 2009). When HSPA5 is co-expressed with MHC-I, the C-terminal region of HSPA5 may be recognized as an autoantigen by the immune surveillance while co-expression with MTJ-1 leads to the avoidance of the immune response. In addition to CV-A9, HSPA5 has been suggested to serve as a coreceptor for dengue virus serotype 2 and bornavirus (Honda et al., 2009, Jindadamrongwech et al., 2004, Triantafilou et al., 2002 and 2001).

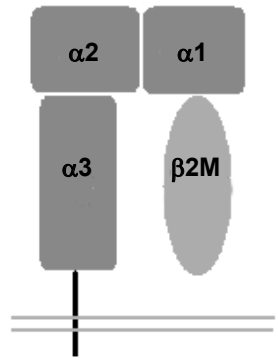


**Figure 8**. The structure of MHC class I molecule. MHC-I contains one heavy chain (α1-α3) and one light chain (β2M). α1 and α2 participate in peptide binding while the immunoglobulin-like α3 is anchored to the plasma membrane. (Modified from Natarajan et al., 1999).

The involvement of heparan sulfate/heparin class of proteoglycans (HSPG) in CV-A9 binding has also been suggested. Heparan sulfate (HS) has ubiquitous distribution on cell surfaces and in extracellular matrix and it participates in numerous biological/ cellular processes, for example in cytokine action, cell adhesion and regulation of enzyme catalysis (Salmivirta et al., 1996). In addition, heparan sulfate has been shown to bind certain picornaviruses such as echovirus 5 (E-5) and specific FMD viruses on the cell surface albeit the interaction does not induce signals for endocytosis and therefore it serves mainly as an attachment factor for viruses (Israelsson et al., 2010, Baranowski et al., 2000). In a recent study, McLeish et al. (2012) have reported that two CV-A9 isolates with an arginine at position 132 in VP1 (T132R) rather than a threonine seen in the non-binding strains (e.g. Griggs), can bind to heparan sulfate suggesting that membrane-associated HSPG can serve as a receptor or coreceptor for CV-A9. They suggest that CV-A9 has a specific HS-binding site that may be common between enterovirus types. Contrary to their results, recent findings from our laboratory indicate that the prototype strain of CV-A9 (Griggs) is fully capable of binding heparan sulphate. In addition, the putative HS-binding site was not detected in fifty clinical CV-A9 isolates suggesting that it is not important in natural CV-A9 infections (Karelehto et al. 2013, manuscript in preparation).

Very little is known about the endocytosis mechanisms of coxsackievirus A9 despite the fact that the complete genome and capsid structure of CV-A9 have been resolved more than ten years ago. The involvement of β2M in CV-A9 endocytosis seems to be important since it has been shown that blocking of β2M prevents the infections of several other enteroviruses as well (Marjomäki et al., 2002, Ward et al., 1998). In addition, the role of HSPA5 has been suggested to be essential since integrin binding alone does not trigger the endocytosis of the virus and the infection is not dependent on integrin interaction in all cell lines. Triantafilou et al. (2003 and 2002) have proposed a model for CV-A9 endocytosis where CV-A9 utilizes αVβ3 integrin as its primary attachment receptor and HSPA5 as its coreceptor on a cell surface. They suggest that interaction with HSPA5 cross-links HSPA5 and MHC-I concentrating these molecules to the same location on the cell surface. This initiates the internalization and endocytosis of CV-A9. However, this model does not explain the molecular mechanism and site of action of these molecules. Furthermore, it does not explain the detected RGD- and integrin-independent attachment and endocytosis of CV-A9 for example in RD cell line. Although specific molecules have been identified to play a role in the CV-A9 attachment and internalization, their exact roles and the endocytic pathway of the virus have remained unclear.

# 3 AIMS OF THE STUDY

Three enteroviruses and one parechovirus have been experimentally shown to use integrins as their receptors. In addition, two enterovirus and four parechovirus types possess RGD-motif in a location similar to CV-A9, which implies that they may also use integrins as cellular receptors (Merilahti et al. 2012). Coxsackievirus A9 (CV-A9) binds to integrins αVβ3 and αVβ6 *in vitro* via RGD-motif that resides in the VP1 capsid protein. However, the studies of the integrin role in CV-A9 attachment and endocytosis are partly contradicting and internalization mechanisms that follow after CV-A9 has bound to a cell surface are poorly characterized. Moreover, Hughes et al. (1995) and Roivainen et al. (1996) showed that CV-A9 infects certain cell lines independently of RGD-motif implying that RGD or integrins are not always required for successful CV-A9 infection. Furthermore, Triantafilou et al. (2003 and 2002) proposed that CV-A9 interaction with HSPA5 cross-links HSPA5 and MHC-I, which initiates the internalization and endocytosis of CV-A9.

Thus, the aim of this thesis was to elucidate further the molecules and mechanisms of cell attachment and internalization of CV-A9 in human epithelial lung carcinoma (A549), human rhabdomyosarcoma (RD) and human epithelial colorectal adenocarcinoma (SW480) cell lines, and to establish methodology for generation of GFP-expressing CV-A9 particles for real-time imaging in tissues and in mouse model.

The specific aims of this thesis were:

1. to analyze the role of integrins αVβ3 and αVβ6 in CV-A9 infection
2. to determine the role of RGD-motif in CV-A9 binding and infectivity
3. to identify the RGD-independent attachment receptor(s) for CV-A9
4. to study the mechanisms of RGD-dependent and RGD-independent internalization of CV-A9
5. to develop a GFP-expressing CV-A9 for real-time imaging studies

# 4 MATERIALS AND METHODS

## 4.1 Viruses and virus purification (I-IV)

Wild-type coxsackievirus A9 (CV-A9, Griggs strain; I-III), CV-A9 RGD-mutants CV-A9 RGE (I) and CV-A9 RGDdel (I, II) and adenovirus 5 (Ad5; I) were from laboratory collections (Hendry et al., 1999, Hughes et al., 1995, Chang et al., 1989). Viruses were propagated in human A549 epithelial lung carcinoma (CV-A9 and Ad5) or RD-rhabdomyosarcoma (CV-A9 and CV-A9 mutants) cell lines and purified by sucrose gradient ultracentrifugation as described previously (Abraham and Colonno, 1984).

## 4.2 Cell lines and cultures (I-IV)

Human epithelial lung carcinoma (A549; I-III), rhabdomyosarcoma (RD; I, III), African green monkey kidney (GMK; I, IV), human colorectal adenocarcinoma (SW480; Leibovitz, 1976; III) and human cervical cancer (HeLa; IV) cells were obtained from the ATCC (American Type Culture Collection). The second batch of SW480 cells (III) was obtained from Dr. Stephen Nishimura (UCSF, USA). Caveolin-1-negative human hepatocellular carcinoma cell line (HuH7; Vainio et al., 2002; II) was a gift from Dr. Elina Ikonen (University of Helsinki, Finland) and Phoenix Gag-Pol packaging cell line (http://www.stanford.edu/group/nolan/index.html; II) from Dr. Aki Manninen (University of Oulu) with authorization by Garry Nolan (The School of Medicine, Stanford University, Stanford, CA).

A549 cells were maintained in Ham's F12 or Dulbecco's modified Eagle's media (DMEM) supplemented with 7% fetal calf serum (FCS) and gentamicin. GMK, RD and SW480 cells were grown in DMEM containing 7% FCS and gentamicin and HuH7 and Phoenix cells in DMEM containing 10% FCS and gentamicin. HeLa cells were maintained in BME (Eagle's Basal Medium) supplemented with 7% FCS and gentamicin. For virus infections, the culture media were supplemented with 1% FCS.

Caveolin-1-silenced A549 cell line (II) was generated by using retrovirus-mediated RNAi. A retroviral vector RVH1-cav1-KD-puro (Manninen et al., 2005, Schuck et al., 2004) and plasmid pMD.G expressing the vesicular stomatitis virus G protein (VSV-G) were obtained from Dr. Aki Manninen (University of Oulu, Finland). Recombinant viruses were generated by transfecting Phoenix cells with RVH1-cav1-KD-puro, pMD.G and FuGENE 6 according to manufacturer's instructions. Transfected Phoenix cells were incubated at 37°C and the medium was collected at 2-5 days posttransfection. Retrovirus-containing supernatant was added on to A549 cells and polybrene (hexadimethrine bromide, Sigma- Aldrich) was added to virus preparations prior to use. Retrovirus-transduced cells were selected by puromycin (BD Bioscience) and silencing efficiencies were determined by Western analysis and confocal microscopy.

## 4.3 Proteins, antibodies and cellular markers (I-IV)

Purified integrin αVβ3 was obtained from Chemicon/Millipore (I) or BioMarket Ltd (II) and integrin αVβ6 was produced in Chinese hamster ovary (CHO) cells and purified as described previously (Weinacker et al., 1994; I, III). Integrin α5β1 (III) and β2-microglobulin (III) were obtained from Chemicon/Millipore (catalog items CC1052 and CBL62020).

Polyclonal rabbit antiserum against CV-A9 was produced as described earlier (Pulli et al., 1998) and mouse monoclonal antibody (MAb, K6) against CV-A9 (Buttinelli et al., 2003) was obtained from Dr. Lucia Fiore (Instituto Superiore di Sanita, Rome, Italy). The primary monoclonal antibodies specific to different integrins were against αV (L230, ATCC), αVβ3 (MAB1976Z; Chemicon/Millipore), αVβ6 (MAB2077Z and MAB2074Z; Chemicon/Millipore), αVβ5 (MAB1961Z; Chemicon/Millipore), β1 (MAB2253; Chemicon/Millipore) and integrin α5β1 (MAB1969; Chemicon, Millipore). The rabbit antiserum against integrin αVβ3 was a kind gift from Dr. Merja Roivainen (National Institute of Health and Welfare, Helsinki, Finland). Monoclonal antibody specific to β2-microglobulin came from Santa Cruz Biotechnology (sc-51509) and polyclonal antibody from Chemicon/Millipore (RAB730). MAb against Arf6, and rabbit polyclonal antiserum specific to caveolin-1 and heat shock 70-kDa protein 5 (HSPA5 protein, also known as glucose-regulated protein 78-kDa, or GRP78) were from Santa Cruz Biotechnology (sc-7971, sc-894 and sc-13968, respectively). Rabbit polyclonal hemagglutinin (HA) antiserum was obtained from Zymed Laboratories Inc. (71-5500) and rabbit polyclonal antiserum against Erk1 (sc-94) from Santa Cruz Biotechnology. MAb specific to EEA-1 came from BD Transduction Laboratories™ (610457). Alexa Fluor (AF) 488-, 546-, and 568-labeled anti-mouse and anti-rabbit secondary antibodies came from Molecular Probes, and horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody was obtained from Pierce. In all immunofluorescence experiments, the nuclei were stained with Hoechst 33342 (Sigma-Aldrich). Alexa Fluor (AF) 546-conjugated dextran and transferrin and AF 488-conjugated cholera toxin B were obtained from Molecular Probes.

## 4.4 Virus infection assays (I-IV)

To obtain growth curves, confluent A549 and RD cells were infected with native CV-A9 and RGD-mutants (CV-A9 RGE and CV-A9 RGDdel) at the m.o.i of 1. Viruses were incubated at 37°C for 0-24 h and the samples were collected every 2 hours after which the samples were freeze-thawed three times before plaque assay. In a plaque assay, confluent RD monolayers were infected with viruses at RT for 15 min in Hank's solution. After washing, 0.5 % carboxymethylcellulose (CMC) solution in MEMII medium was added and cells were incubated for 2 days before staining with crystal violet. When plaque assay with soluble integrin αVβ3 and αVβ6 was performed, 1000 PFU of native CV-A9 and 20 ng or 200 ng of integrins were mixed and incubated at 37°C for 1 h and the plaque assay was performed by using confluent GMK cells.

In experiments where the efficiency of virus infection was analyzed by microscopy, the cells were infected with virus dilution aiming at 60% efficiency of infection in untreated cells. Viruses were bound to cells on ice, unbound viruses were removed and the infections were allowed to proceed at 37°C for desired time periods. Then the cells were fixed with 4% formalin and permeabilized with 0.2% Triton X-100 (0 min samples were not permeabilized) following the staining with appropriate antibodies and Hoechst. In the siRNA screen and antibody and peptide blocking assays (see below), the virus dilution was set to achieve 10% efficiency of infection in untreated cells.

## 4.5 Production of viral VP1 proteins and cell adhesion assay (I)

The VP1 region (nt 2448-3353) was isolated from cDNA clones of CV-A9 (Griggs prototype; GenBank accession no. D00627) and CV-A9 RGD-mutants (Hughes et al., 1995) and cloned into pET15b (Novagen) after which the constructs were transformed into T7 Express cells (New England Biolabs). Protein production was induced with 1 mM IPTG for 3 h. The cells were then lysed with Bugbuster lysis buffer (Novagen) containing lysozyme, benzonase and protease inhibitors. Vortexed and centrifuged (10000g, 15 min, 4°C) protein pellets were dissolved in 8 M urea (Tris-buffered saline, 10 mM 2-mercaptoethanol) and dialyzed (PBS supplemented with 1 mM $MgCl_2$). In the cell adhesion assay, the attachment of CV-A9 and RGD-mutants or VP1 proteins to A549 or RD cells was analyzed by coating 96-well plates (Costar High Binding) with viruses or proteins. The plates were incubated over night in cold (+4°C) and then washed with PBS containing $MgCl_2$ and $MnCl_2$. Non-specific binding was blocked by BSA after which the virus-coated wells were overlaid with detached A549 and RD cells. After an incubation at +37°C for 1 h cell attachment was visualized by staining with crystal violet. Absorbance was measured with a spectrophotometer at 590 nm.

## 4.6 Integrin expression, flow cytometry and quantification (I, III)

Expression of integrins (αV, αVβ6, αVβ3, αVβ5, β1 and α5β1) on the cell surface was analyzed by flow cytometry by using specific monoclonal antibodies. Cells were detached and suspended in PBS after which the receptor specific antibodies were added. Cells were incubated at 4°C for 15 min, pelleted, washed once with PBS and suspended in PBS. After washing, the incubation was repeated in the presence of secondary AF-antibodies and after washing, suspended cells (in PBS) were analyzed with FACS Calibur flow cytometer (Becton Dickinson). In each assay, 10 000 cells were analyzed and the data analysis was made by cyflogic program (version 1.1.0; http://www.cyflogic.com/). When the total gene expression levels of integrin subunits (αV, β6, β3 and β1) were analyzed by quantitative reverse transcription-PCR (qRT-PCR), the mRNA was isolated from 300 000 cells with PolyATract system 1000 (Promega) according to the manufacturer's instructions. The cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) and oligo(dT)15 primer (Finnzymes)

and by incubating the mRNA template at 37°C for 60 min. The real-time PCR was done with Maxima SYBR green qPCR Master Mix (Fermentas) using a QuantiTect Primer Assay (Qiagen) for each studied gene. The qPCR run was performed with a RotorGene 6000 real-time PCR cycler (Corbett). The expression levels of β2M were used to standardize the qRT-PCR results.

## 4.7 Blocking and binding assays (I-III)

In an antibody blocking assay, the blocking effects were analyzed in a plaque assay or by measuring the fluorescence intensities with Victor3 multilabel reader (Perkin Elmer). When plaque assay was used (I), function-blocking integrin antibodies against αV, αVβ3, αVβ5 and β1 were added onto confluent RD cells. The antibodies were removed and the cells were infected with CV-A9 or CV-A9 RGDdel. After washing and incubation steps, the cells were detached and freeze-thawed three times before plaque assay that was performed in GMK cells. When Victor3 was used (III), confluent A549 or SW480 cells were overlaid with function-blocking integrin antibodies against αV, αVβ5 and β1 or MAb against β2-microglobulin. After unbound antibodies were removed, CV-A9 was added and the infection was followed for 6 h before cell fixation, permeabilization and staining with the virus specific and secondary AF 488-labeled antibodies. Nuclei were stained with Hoechst and the ratio of virus specific signal to the total cell number was determined by Victor3.

The binding of CV-A9 and CV-A9 RGD-mutants to integrin αVβ3 and αVβ6 and CV-A9 binding to integrin α5β1 were analyzed in solid-phase integrin binding assay where the wells (Costar High Binding) were first coated with integrins and then the virus dilutions were added onto wells. Viruses that bound to integrins were stained with the virus specific monoclonal or polyclonal antibody and, after incubation and washing steps, secondary anti-rabbit or anti-mouse horseradish peroxidase conjugate (HRP, Pierce) was added. The wells were stained with $H_2O_2$, incubated at dark and the reaction was stopped with 0.45 M $H_2SO_4$. The absorbance was read at 450 nm. The same protocol was used when integrin binding to immobilized viruses was determined, but then the wells were coated with viruses and overlaid with integrins. For the detection, the unblocking antibodies against αVβ6 and αVβ3 were used.

CV-A9-binding peptides were selected from a phage display library (III; a gift from Dr. Erkki Koivunen, University of Helsinki, Finland) that contained eight amino acid long cyclic peptides inserted in the pIII capsid protein in the M13 phage (Koivunen et al., 1999). CV-A9 was bound to microtiter wells and the phage solution was then transferred to the virus-coated wells. Unbound phages were removed with Tris-buffered saline containing 0.5% Tween 20 and the bound phages were eluted with 0.1 M HCl (pH 2.2) in the presence of BSA. The eluted phages were neutralized with 1 M Tris-HCl and amplified using K91kan bacteria after which they were precipitated with polyethylene glycol. The panning procedure was repeated twice and the phage DNAs were purified

and sequenced. Peptide sequences found in the library were compared against protein sequences in the SwissProt data base using the FASTA program. In a peptide blocking assay, peptide ESPLSLVA found in the phage library and a negative control RRRGEL were used. Peptides were added onto SW480 cell monolayer after which the cells were incubated on ice for 30 min before virus attachment in the presence of peptides. After the unbound virus was removed, the infection was followed first for 6 h at +37°C and then the cells were fixed, permeabilized and stained with the CV-A9 specific and secondary antibodies. Nuclei were stained with Hoechst and the virus and Hoechst signals were measured with Victor3 multilabel counter.

## 4.8 Plasmids, transfection and transduction with adenoviral vectors (II)

GFP-dynamin 2aa and GFP-dynamin 2aaK44A expression plasmids (Ochoa et al., 2000, Cao et al., 1998) for wild-type (wt) and dominant-negative (DN) dynamin 2 were obtained from Mark McNiven (Mayo Clinic, Rochester, MN). HA-tagged caveolin-3 and caveolin-3DGV expression plasmids for wt and DN caveolin-3 (Roy et al., 1999) were received from Robert Parton (The University of Queensland, Brisbane, Australia) while Eps15-GFP and Eps15E_95/295-GFP constructs (Benmerah et al., 1999) for wt and DN Eps15 were from Alice Dautry-Varsat (Pasteur Institute, Paris, France) and DN AP180C construct (Ford et al., 2001) from Dieter Blaas (University of Vienna, Austria). Plasmids pcDNA3 HA Arf6 and pcDNA3 HA Arf6 DN T27N (Furman et al., 2002) for the expression of wt and DN Arf6 were from Addgene (10834 and 10831, www.addgene.org) and recombinant GFP-tagged Eps15-adenoviral vectors Ad:DIII and rAd:DIII_2 (Lecot et al., 2005) for wt and DN Eps15, respectively, were provided by Yves Rouille` (Pasteur Institute, Lille, France).

A549 cells were cultured up to 50% confluency and transfected with expression plasmids using FuGENE 6 or FuGENE HD (Roche) according to manufacturer's protocol. The adenovirus vectors were produced in HEK293T cells and used to transduce semiconfluent A549 cells using standard methods. The CV-A9 infection assay was done at 48 h after plasmid transfection or adenovirus transduction.

## 4.9 Chemical inhibitors and drug treatments (II-III)

In the inhibitor assay, A549 (II) or SW480 (III) cells were preincubated for 30 min at 37°C with several chemical inhibitors before addition of CV-A9. The inhibitors were present throughout the experiments. The unbound virus was removed and the virus infection was followed for 6 h at +37°C before cell fixation, permeabilization and staining with the virus-specific antibody, secondary antibody and Hoechst. The inhibitors used in the work were as follows: 100 or 150 μM 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA; A3085, Sigma), 25 or 50 μM chlorpromazine (C8138, Sigma), 1 mM methyl-β-cyclodextrin (MβC]; C4555, Sigma), 80 μM dynasore (D7693, Sigma), 33.2 μM nocodazole (M1404, Sigma), 5 or

50 µg/ml cytochalasin D (C8273, Sigma), 2 µM jasplakinolide (420127, Calbiochem), 1 µM latrunculin A (L5163, Sigma), 100 or 400 nM wortmannin (W1628, Sigma), 5 µg/ml filipin (F4767, Sigma) and the combination of 25 µg/ml nystatin (N3503, Sigma) and 10 µg/ml progesterone (P8783, Sigma). The importance of endosomal acidification was studied with 0.5 mM, 2 mM, 5 mM, or 25 mM $NH_4Cl$. AF488-conjugated cholera toxin B (0.2 µg/ml) and AF 546-conjugated transferrin (10 µg/ml) were used to control the function of chlorpromazine, MβC and dynasore, while AF 546-conjugated dextran (250 µg/ml) was used to control the function of EIPA and cytochalasin D. After incubation of cells with inhibitors, the cell markers were added and incubation was continued for 15 min at 37°C before cell fixation and confocal imaging.

## 4.10 siRNA methods (II-III)

In the siRNA assays, two individual siRNA molecules (Qiagen) for each gene were used (**Tables 2 and 3**). For transfection of A549 and SW480 cells, 0.5-1 pmol siRNA in 25 µl $H_2O$ was mixed with 0.2-0.4 µl siLentFect (Bio-Rad) diluted in serum free medium and the wells were incubated at RT for 30 min after which 13 000 (A549) - 25 000 (SW480) cells were added and cultured for two days at 37°C prior to virus infection and staining. The fluorescence intensities were measured with a Victor3 multilabel reader. Transfection conditions were optimized by transfecting the cells with siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and measuring the GAPDH enzyme activity with a KDalert GADPH Assay Kit (Applied Biosystems). Silencing efficiencies were measured by quantitative reverse transcription-PCR (qRT-PCR) or Western analysis. The cell viability after 48 h of siRNA transfection was tested by double staining the cells with the dead-cell marker Sytox Orange nucleic acid stain (Molecular Probes) and Hoechst after which the fluorescence intensities were measured.

**Table 2.** Receptor siRNAs used in this study.

| Gene | Protein, aliases | siRNA |
|---|---|---|
| | Negative control siRNA | AllStars |
| **PVR** | CD155, PVR | Hs_PVR_4<br>Hs_PVR_5 |
| **ICAM1** | ICAM-1, Intracellular adhesion molecule-1, CD54 | Hs_ICAM1_6<br>Hs_ICAM1_7 |
| **CXADR** | CAR, Coxsackievirus-adenovirus receptor | Hs_CXADR_9<br>Hs_CXADR_11 |
| **HAVCR1** | HAVcr-1; HAV cellular receptor | Hs_HAVCR1_8<br>Hs_HAVCR1_9 |
| **CD55** | DAF, decay accelerating factor, CD55 | Hs_CD55_2<br>Hs_DAF_3 |
| **ITGB1** | Integrin β1 | Hs_ITGB1_5<br>Hs_ITGB1_9 |
| **ITGB3** | Integrin β3 | Hs_ITGB3_1<br>Hs_ITGB3_5 |
| **ITGB6** | Integrin β6 | Hs_ITGB6_1<br>Hs_ITGB6_5 |
| **ITGB8** | Integrin β8 | Hs_ITGB8_5<br>Hs_ITGB8_6 |
| **ITGA2** | Integrin α2 | Hs_ITGA2_5<br>Hs_ITGA2_6 |
| **ITGA5** | Integrin α5 | Hs_ITGA5_5<br>Hs_ITGA5_7 |
| **LDLR** | LDL-R, Low-density lipoprotein receptor | Hs_LDLR_3<br>Hs_LDLR_4 |
| **NANS** | N-acetylneuraminic acid synthase (sialic acid synthase), SAS | Hs_NANS_2<br>Hs_NANS_5 |
| **EXT1** | exostoses (multiple) 1; (an ER-resident type II transmembrane glycosyltransferase involved in the chain elongation step of heparan sulfate biosynthesis) | Hs_EXT1_1<br>Hs_EXT1_4 |
| **B2M** | Beta-2-microglobulin | Hs_B2M_3<br>Hs_B2M_4 |

**Table 3.** Other siRNAs used in this study.

| Gene | siRNA | Gene | siRNA |
|---|---|---|---|
| **Caveolin 1** | Hs_CAV1_6<br>Hs_CAV1_7 | **Mitogen-activated protein kinase 8** | Hs_MAPK8_9<br>Hs_MAPK8_13 |
| **Dynamin 2** | Hs_DNM2_8<br>Hs_DNM2_3 | **Jun oncogene** | Hs_JUN_5<br>Hs_JUN_3 |
| **Early endosome antigen 1** | Hs_EEA1_1<br>Hs_EEA1_4 | **Mitogen-activated protein kinase 3** | Hs_MAPK3_7<br>Hs_MAPK3_4 |
| **Clathrin, heavy chain** | Hs_CLTC_9<br>Hs_CLTC_10 | **FBJ murine osteosarcoma viral oncogene homolog** | Hs_FOS_6<br>Hs_FOS_5 |
| **RAB5A, member RAS oncogene family** | Hs_RAB5A_5<br>Hs_RAB5A_8 | **Ras homolog gene family, member A** | Hs_RHOA_7<br>Hs_RHOA_6 |

| Gene | siRNA | Gene | siRNA |
|---|---|---|---|
| **RAB4A, member RAS oncogene family** | Hs_RAB4A_5<br>Hs_RAB4A_6 | **Protein kinase, cAMP-dependent, catalytic, α** | Hs_PRKACA_6<br>Hs_PRKACA_5 |
| **RAB7A, member RAS oncogene family** | Hs_RAB7_5<br>Hs_RAB7_6 | **Protein kinase C, α** | Hs_PRKCA_5<br>Hs_PRKCA_7 |
| **RAB9A, member of RAS oncogene family** | Hs_RAB9A_5<br>Hs_RAB9A_4 | **Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3** | Hs_DYRK3_10<br>Hs_DYRK3_9 |
| **AP2 associated kinase 1** | Hs_AAK1_5<br>Hs_AAK1_6 | **Heat shock 70kDa protein 5** | Hs_HSPA5_6<br>Hs_HSPA5_7 |
| **ADP-ribosylation factor 1** | Hs_ARF1_8<br>Hs_ARF1_11 | **BR serine/threonine kinase 1** | Hs_BRSK1_1<br>Hs_BRSK1_2 |
| **Transient receptor potential cation channel, subfamily M, member 6** | Hs_TRPM6_3<br>Hs_TRPM6_1 | **Pumilio homolog 1** | Hs_PUM1_1<br>Hs_PUM1_2 |
| **Talin 1** | Hs_TLN1_5<br>Hs_TLN1_4 | **COP9 constitutive photomorphogenic homolog subunit 5** | Hs_COPS5_3<br>Hs_COPS5_7 |
| **Phosphoinositide-3-kinase, regulatory subunit 1** | Hs_PIK3R1_5<br>Hs_PIK3R1_6 | **v-src sarcoma viral oncogene homolog** | Hs_SRC_7<br>Hs_SRC_6 |
| **v-akt murine thymoma viral oncogene homolog 1** | Hs_AKT1_5<br>Hs_AKT1_7 | **Integrin-linked kinase** | Hs_ILK_4<br>Hs_ILK_7 |
| **PTK2 protein tyrosine kinase 2** | Hs_PTK2_9<br>Hs_PTK2_5 | **Mitogen-activated protein kinase kinase kinase 2** | Hs_MAP3K2_7<br>Hs_MAP3K2_5 |
| **v-Ha-ras Harvey rat sarcoma viral oncogene homolog** | Hs_HRAS_7<br>Hs_HRAS_8 | **Mitogen-activated protein kinase 14** | Hs_MAPK14_6<br>Hs_MAPK14_7 |
| **Ras-related C3 botulinum toxin substrate 1** | Hs_RAC1_6<br>Hs_RAC1_7 | **Mitogen-activated protein kinase kinase kinase 8** | Hs_MAP3K8_7<br>Hs_MAP3K8_5 |
| **p21 protein (Cdc42/Rac)-activated kinase 1** | Hs_PAK1_8<br>Hs_PAK1_7 | **Mechanistic target of rapamycin (serine/threonine kinase)** | Hs_FRAP1_5<br>Hs_FRAP1_6 |
| **Cell division cycle 42 (GTP binding protein, 25kDa)** | Hs_CDC42_7<br>Hs_CDC42_12 | **Twinfilin** | Hs_PTK9_5<br>Hs_PTK9_9 |
| **FYN oncogene related to SRC, FGR, YES** | Hs_FYN_5<br>Hs_FYN_7 | **v-rel reticuloendotheliosis viral oncogene homolog A (avian)** | Hs_RELA_5<br>Hs_RELA_7 |
| **Cysteine-rich, angiogenic inducer, 61** | Hs_CYR61_7<br>Hs_CYR61_8 | **Growth arrest and DNA-damage-inducible, β** | Hs_GADD45B_2<br>Hs_GADD45B_5 |
| **jun B proto-oncogene** | Hs_JUNB_2<br>Hs_JUNB_5 | **CDC-like kinase 1** | Hs_CLK1_5<br>Hs_CLK1_6 |
| **Early growth response 1** | Hs_EGR1_6<br>Hs_EGR1_7 | **ADP-ribosylation factor 6** | Hs_Arf6_5<br>Hs_Arf6_7 |
| **pim-1 oncogene** | Hs_PIM1_6<br>Hs_PIM1_2 | **Glyceraldehyde-3-phosphate dehydrogenase** | Hs_GAPDH_3 |

## 4.11 Endocytosis, imaging and data analysis (II-IV)

In all endocytosis assays, A549 or SW480 cells were first grown on cover slips at +37°C overnight and then transferred onto ice. Virus infection was performed as described above and the infection was followed at 37°C for 0 min to 6 h depending on assay after which the cells were fixed, permeabilized (excluding 0 min) and analyzed by imaging with fluorescence microscopy (Zeiss Axiovert 200 M) or confocal microscopy (Zeiss

LSM510 META). When the internalization of CV-A9 was followed simultaneously with AF 546-conjugated transferrin and AF 546-conjugated dextran, the virus was first attached onto cells, then the cell markers were added and the infection was followed for desired time periods. When colocalization between CV-A9 and integrin αVβ6, EEA1, β2M, HSPA5 and Arf6 were analyzed, cells were stained with appropriate antibodies. All antibodies were diluted in PBS supplemented with 3% BSA. After the incubation with primary and secondary antibodies, the nuclei were stained with Hoechst and the cells were mounted on microscope slides in mowiol 4-88 (Calbiochem-Novabiochem) prepared in 25% glycerol, 0.1 M Tris-HCl, pH 8.5 and 25 mg/ml DABCO (Sigma-Aldrich). When the data was examined with confocal microscopy, Plan-Apochromat objective (63x oil / 1.4) was used. All images were converted to TIFF format and slightly formatted in ImageJ (http://rsb.info.nih.gov/ij), Photoshop CS3 program, BioImageXD software (Kankaanpää et al., 2012) or AxioVert LE v4.5.

## 4.12 Long PCR and modification of CV-A9 cDNA clone (IV)

CV-A9 genome (Griggs prototype; GenBank accession no. D00627) cloned under T7 promoter was from the laboratory collection and used with kind permission from Dr. Glyn Stanway (University of Essex, UK; Hughes et al., 1995). The plasmid pCMV-T7 pol encoding functional T7 RNA polymerase was a gift from Ralph Meyer (University Hospital of Tübingen, Germany) and has been described previously (Meyer et al., 2004). Two back-to-back primers (CV-A9mut2-F: 5′-TTGGCCAGCAGGGCGGCCGCCTTAACACGCATGGTGCCTTCGGACAACAATC-3′ and CV-A9mut2-R: 5′-AAGCGCCGTGGGTATTCAGGGTGGACATGTCACCCCGACGCC-3′) were designed and used in a long PCR reaction utilizing Phusion DNA Polymerase (Finnzymes, Espoo, Finland) to amplify the entire plasmid pCV-A9. In the PCR cycle, 3 min initial denaturation was performed at 98°C, 25 cycles with 30 s denaturation at 98°C and 10 min annealing and extension at 72°C and a final extension at 72°C for 10 min after which the product was ethanol-precipitated and purified (NucleoSpin Extract II, Macherey-Nagel, Düren, Germany). To produce a circular pCV-A9ins plasmid, the linear product was treated with T4 polynucleotide kinase and ligated with T4 ligase (Fermentas, Vilnus, Lithuania) and then the restriction fragment of eGFP was cloned into pCV-A9ins to produce the plasmid pCV-A9-eGFP. The full-length CV-A9 PCR product with 5′ terminal T7 promoter region was generated by using 5′-end primer containing T7 promoter region and 3′-end primer producing a poly-A tail of 20 bases (PCR cycle: 5 min at 98°C, 25 cycles with 30 s at 98°C and 5 min 30 s at 72°C, 10 min at 72°C) after which full-length CV-A9 PCR product was co-transfected into GMK cells with the plasmid encoding T7RNA polymerase. Transfections were performed with Fugene 6. To verify the success of transfections, viral RNA was extracted from transfected cells (QIAamp Viral RNA Mini Kit, Qiagen) and RT-PCR was performed using enterovirus-specific primers (Lönnrot et al., 1999). CV-A9 production and GFP expression were analyzed by fluorescence microscopy as described above (4.11) using CV-A9-specific antibodies.

# 5 RESULTS

## 5.1 Cell surface interactions of coxsackievirus A9 (I-III)

In several studies, coxsackievirus A9 (CV-A9) has been shown to interact with integrins αVβ3 and αVβ6 via an arginine-glycine-asparagine (RGD) motif that resides in the C-terminus of the viral VP1 capsid protein (Williams et al., 2004, Roivainen et al., 1994 and 1991, Chang et al., 1989). However, it has also been shown that CV-A9 mutants that lack RGD-motif infect certain cell lines (Triantafilou et al., 2000, Roivainen et al., 1996, Hughes et al., 1995). The purpose of this work was to make a collective study of the functions of integrins and RGD-motif in CV-A9 infection and to alleviate some of the contradicting results of previous reports about the role of CV-A9 RGD-motif in integrin binding and viral infection (Williams et al., 2004, Triantafilou et al., 2000, Hughes et al., 1995). In addition, the purpose was to identify the integrin- and RGD-independent binding receptor for CV-A9.

### 5.1.1 RGD-dependent and RGD-independent attachment (I-III)

When the infectivity of CV-A9 was analyzed in A549 and RD cells (**I / Fig. 1A and B**), the native CV-A9 was capable of infecting both cell lines but the RGD-mutants, CV-A9 RGE and CV-A9 RGDdel, could only infect RD cells suggesting that RGD-motif is important for virus attachment and infection in A549 cells while in RD cells the binding and entry are RGD-independent processes. This was in agreement with the results of Triantafilou et al. (2000), Roivainen et al. (1996) and Hughes et al. (1995). However, in contrast to the previous studies, CV-A9 and CV-A9 RGDdel were observed to infect SW480 cell line (**III / Fig. 1A)**, which does not express integrin αVβ3 and αVβ6 (Berryman et al., 2005, Williams et al., 2004, Agrez et al., 1994).

To elucidate the differences in the integrin expression patterns on these three cell lines, flow cytometric analysis using antibodies specific to αV, αVβ3, αVβ5, αVβ6 and β1 was performed. The expression of αVβ3 integrin was similar in A549 and RD cells, but integrin αVβ6 was only detected on the surface of A549 cells (**I / Fig. 2A and B)**. This supported the idea that integrin αVβ6 serves as the primary attachment receptor for CV-A9 in RGD-dependent cell lines such as A549 (Williams et al., 2004). As expected, integrins αVβ3 or αVβ6 were not detected on SW480 cells (**III / Fig. 1B**; Berryman et al., 2005, Agrez et al., 1994). The expression patterns of αVβ5, αV and β1 were similar in all cell lines. In SW480 cells, the gene expression levels of β3 and β6 subunits were quantified by RT-qPCR to confirm the lack or low expression levels of αVβ3 and αVβ6 and the results were compared with the integrin expression levels of A549 cells. β3 subunit level was only 22% and β6 subunit level 9% of the level of A549 cells (**III / Fig. 1B**) explaining why the receptors were not detectable in flow cytometry. In addition to αV-integrins, the expression level of another RGD-binding integrin, α5β1, was studied

in SW480 cells (**III / Fig. 2C**). Although α5β1 integrin was clearly expressed on SW480 cells, CV-A9 did not show *in vitro* affinity to it, in contrast to αVβ3 and αVβ6 (**I / Fig. 4A and B; III / Fig. 2D**). These data indicated that CV-A9 attachment to SW480 cells is RGD-independent.

Function-blocking monoclonal antibodies (MAbs) known to interact with their physiological ligands were further used to verify that RGD-binding receptors are not required for the CV-A9 infection cycle in RD cells (**I / Fig. 1C**) and SW480 cell line (**III / Fig. 2A and B**). In RD cells, none of the used antibodies against αV, αVβ3, αVβ5 and β1 prevented the attachment of the CV-A9 RGDdel mutant, and although decreased infection was detected with native CV-A9, the virus was still infectious in these cells. As expected, in SW480 cells none of the used antibodies (αV, αVβ5, β1) blocked the infection. Altogether these data confirm that in A549 cells, the CV-A9 attachment is integrin-dependent and occurs via RGD-motif while in RD and SW480 cells the binding occurs independently of integrins and RGD-motif.

### 5.1.2 Integrin αVβ6 as a high-affinity RGD-binding receptor (I-II)

Since the RGD-motif mediates CV-A9 attachment through an integrin receptor in A549 cells, the affinity and avidity of integrins αVβ3 and αVβ6 were compared. To investigate the efficiency of binding, the native CV-A9 and its deletion mutants, CV-A9 RGE and CV-A9RGDdel, as well as purified RGD-containing viral VP1 capsid proteins were immobilized and overlaid with A549 and RD cells (**I / Fig. 3**). The results showed that A549 cells adhered efficiently to CV-A9 but not to the CV-A9 RGD mutants whereas the adhesion was significantly lower when immobilized viruses were overlaid with RD cells. VP1 proteins did not adhere to either cell line indicating that individual VP1 proteins alone are not sufficient for virus attachment, and hence additional sites on the virus surface may contribute the overall binding to the integrins. VP1 proteins were thought to be functionally active since similarly purified adenovirus 5 penton protein known to adhere to A549 cells gave an efficient binding effect in the assay.

For testing of avidity, integrins αVβ3 and αVβ6 were immobilized and overlaid with CV-A9 (**I / Fig. 4A and B**). The results showed that the avidity of both integrins to CV-A9 was similar but, in contrast, when the affinity test was performed by immobilizing the virus and overlaying with integrins, a clear difference in affinity was detected: αVβ6 bound efficiently to CV-A9 while the affinity to virus was not observed with αVβ3 (**I / Fig. 4C**) suggesting that integrin αVβ6 mediates the high-affinity binding to CV-A9. To confirm these results, soluble integrins αVβ3 and αVβ6 were incubated with CV-A9 and virus infectivity was tested by plaque assay (**I / Fig. 5**). As a result, αVβ6 efficiently inhibited the infection of CV-A9 while αVβ3 rather enhanced the infection. In addition, the silencing of β6 subunit efficiently blocked virus infection whereas silencing of β3 subunit had no effect (**II / Fig. 3A**). These data suggest that in an RGD-dependent cell line integrin αVβ6 is essential and serves as the high-affinity binding receptor for CV-A9.

### 5.1.3 Role of β2-microglobulin and HSPA5 (II-III)

To find the receptor that mediates RGD-independent entry, a siRNA panel against known picornaviral receptor proteins was designed (**Table 2**). The most effective inhibition of CV-A9 infection in SW480 cells was observed with the siRNA targeted against β2-microglobulin (**III / Fig. 3A**), a subunit of major histocompatibility complex class I (MHC-I) complex. In addition, the siRNA against β2-microglobulin (β2M) inhibited the infection also in A549 cells. (**II / Fig. 3A**). However, none of the RGD-binding integrins had an effect on CV-A9 infectivity in SW480 cells while in A549 cell line the silencing of integrin αVβ6 blocked CV-A9 proliferation. To exclude the possibility that the blocking effects were due to cytotoxicity, cell viabilities after siRNA transfections were checked in all siRNA assays (**II / Fig. 3B and III / Fig. 3B**). Because β2M was the only "hit" in the siRNA panel in SW480 cells, its role as an attachment receptor was further analyzed. Firstly, the blocking effect was verified by the antibody blocking assay (**III / Fig. 3C**) and secondly, direct binding assay between β2M and CV-A9 or CV-A9 RGDdel was performed (**III / Fig. 3D**). Although anti-β2M antibody effectively blocked CV-A9 infection in both A549 and SW480 cell lines, direct interaction between the virus and β2M was not detected. In addition, when the virus and β2M were double-labelled on A549 and SW480 cell surfaces, clear colocalization was not observed (Time point 0 min in **II / Fig. 8 and III / Fig. 5B**) suggesting that β2M has a role in a postattachment stage of CV-A9 internalization but it does not serve as an attachment receptor for CV-A9 in either cell line.

Phage display library screening was used to identify novel RGD-independent receptors for CV-A9. In this library, cyclic, degenerate CX8C peptides were displayed on the surface of a filamentous phage particle (Koivunen et al., 1999). Although several peptide binders were identified during multiple panning rounds (**III / Table 1)**, only one of them was of interest because it was identical to the N-terminal sequence of HSPA5 (GRP78) protein (NM_005347.4). The other peptides did not align to known cell surface receptor molecules and, therefore, were not analyzed further in this study. HSPA5 protein is an endoplasmic reticulum chaperone but it is also expressed on the surface of various cell types (Gonzalez-Gronow et al., 2009, Triantafilou et al., 2002). Triantafilou et al. (2002) were the first to suggest that HSPA5 protein serves as a coreceptor for CV-A9. To analyze whether the peptide ESPLSLVA identified by using the phage library screening prevents virus infection in SW480 cells, the peptide blocking assay was performed (**III / Fig. 4**). As a result, CV-A9 infection was completely blocked by the peptide suggesting that HSPA5 has a significant role in CV-A9 attachment on the cell surface. In addition, the same result was observed in A549 cells (unpublished data) indicating that HSPA5 is important in CV-A9 attachment. When CV-A9 and HSPA5 protein were double-labelled on SW480 cell surface, significant colocalization (29%) was detected (time point 0 min in **III / Fig. 5C**). These data demonstrate that HSPA5 serves as a receptor for CV-A9 in SW480 cells regardless of RGD-motif or the presence of RGD-binding integrin(s) and is likely to mediate CV-A9 infection in A549 cells as well.

## 5.2 Endocytosis of coxsackievirus A9 (II-III)

The steps that follow after coxsackievirus A9 has attached to the cell surface integrin or other receptor have been poorly characterized. An early electron microscopy study showed that CV-A9 particles entered monkey kidney cells in vesicles that fuse and form large structures (Hecker et al., 1974) and eventually, internalized particles became trapped in large vacuoles that may be lysosomes. More recently, it was proposed that CV-A9 enters the cell through lipid microdomains and that the molecules that participate in virus internalization are β2-microglobulin and HSPA5 protein (GRP78; Triantafilou and Triantafilou 2003; Triantafilou et al., 2002). In this work, the internalization mechanisms were studied in RGD-dependent A549 and RGD-independent SW480 cell lines in order to demonstrate whether CV-A9 utilizes different virus entry pathways depending on the cell type.

### 5.2.1 RGD-dependent entry (II)

#### 5.2.1.1 αVβ6 integrin functions in coxsackievirus A9 attachment but not in entry

In order to demonstrate whether CV-A9 particles and αVβ6 integrins generate clusters on A549 cell surface and penetrate the cell membrane in close contact, the virus and integrin were double-stained and analyzed by confocal microscopy. However, clear colocalization was not detected at 0 min, 5 min or 20 min postinfection (**II / Fig. 4**), which indicated that αVβ6 integrin does not mediate the endocytosis of the virus. Silencing of integrin signaling molecules Src, Fyn, RhoA, PI(3)K and Akt1 did not alter the infection of CV-A9 either (**II / Fig. 3A**) suggesting that the integrin-linked signaling is not required for CV-A9 entry in A549 cell line. The silencing results were supported by the finding that the PI(3)K inhibitor wortmannin did not inhibit CV-A9 infection (**II / Fig. 1A**). These data implied that although integrin αVβ6 is crucial for CV-A9 infection in A549 cells, the virus does not form clusters and internalize with the integrin, and that the integrin-linked signaling does not play a role in virus entry. Thus, the data suggest that αVβ6 serves as a cell attachment factor and other factors are required for efficient CV-A9 entry.

#### 5.2.1.2 Role of macropinocytosis in coxsackievirus A9 internalization

In order to elucidate the endocytic pathway utilized by CV-A9, the effects of chemical inhibitors (functions described in **II / Table 1**) of endocytosis were studied. A significant reduction in virus infection was observed with EIPA, which inhibits $NA^+/H^+$ exchange and with jasplakinolide, which stabilizes actin microfilaments (**II / Fig. 1A**). The effect of EIPA was further studied since EIPA has a potential role in inhibition of macropinocytosis (Mercer and Helenius, 2009). In confocal microscopy analysis, virus infection was observed to be arrested in EIPA-treated cells and virus particles were seen in clusters or in vesicle-like structures close to the cell periphery (**II / Fig. 2A)**. A similar effect was also detected with echovirus 1 (Karjalainen et al., 2008). However, since the function of EIPA has been shown to be cell type-dependent and non-specific (Fretz et al., 2006), the involvement

of macropinocytosis in CV-A9 infection cycle was studied in a co-internalization assay with the virus and macropinocytic marker dextran (**II / Fig. 2C**). As a result, dextran entered A549 cells clearly faster than CV-A9 suggesting that the virus does not utilize macropinocytosis when entering the cell. Although jasplakinolide significantly reduced virus infection, the actin-disrupting agents, cytochalasin D and latrunculin A (**II / Fig. 1A**) did not inhibit CV-A9 proliferation, which contradicts the involvement of macropinocytosis in virus entry. Furthermore, silencing of macropinocytosis-regulatory molecules (PI(3)K, Rac1, Pac1, Cdc41 and Rab5) did not reduce CV-A9 proliferation (**II / Fig. 3A and Table 3 in Materials and methods**). These results indicate that CV-A9 internalization is not dependent on macropinocytosis in A549 cell line.

#### 5.2.1.3 Coxsackievirus A9 does not utilize clathrin- or caveolin-1-mediated endocytosis in A549 cells

Since the common markers of macropinocytosis were not found to be involved in CV-A9 cell entry, the involvement of other central endocytic molecules, clathrin and caveolin were studied. The chemical inhibitor chlorpromazine that inhibits clathrin-dependent endocytosis did not reduce virus infectivity (**II / Fig. 1A**) suggesting that the clathrin-dependent route does not play a role in the internalization. This result was confirmed by the observation that two DNA constructs of clathrin route markers, Eps15EΔ95/295 and AP180C did not affect viral infection and the same result was obtained with cells transduced with DN Eps15-adenovirus vector (**II / Fig 5A and B**). In addition, neither of the used siRNAs against genes involved in clathrin-mediated endocytosis (EEA1, Rab5, AAK1, clathrin heavy chain) prevented virus infection (**II / Fig. 3A**). Since dependence on acidic pH may indicate that a virus utilizes clathrin-mediated pathway (Pelkmans and Helenius, 2003), virus infection was followed in the presence of $NH_4Cl$. However, CV-A9 was not sensitive to the inhibition of endosomal acidification (**Figure 9**) supporting the assumption that CV-A9 does not enter the A549 cell via the clathrin route.

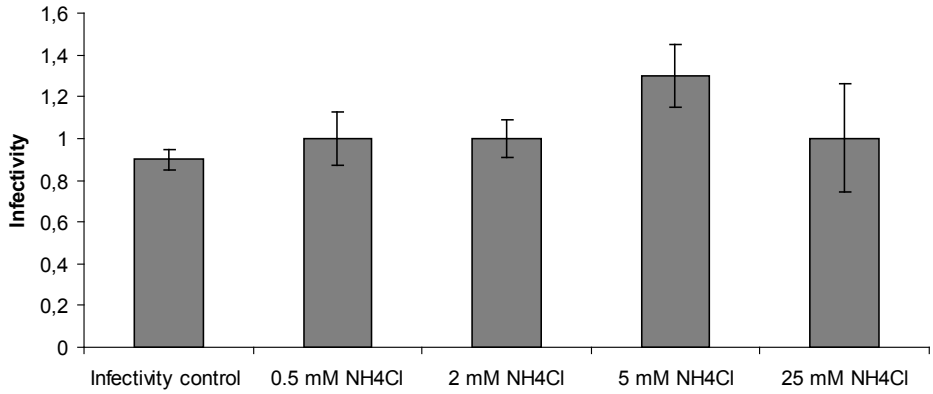


**Figure 9.** Coxsackievirus A9 is not sensitive to the inhibition of endosomal acidification in A549 cells. Virus infection was followed for 6 h in the absence (infectivity control) or the presence of variable concentrations of $NH_4Cl$ before cell fixation, virus staining and fluorescence measurement. Error bars indicate standard deviation from five independent experiments.

Caveolin-mediated endocytic route was blocked by using a combination of chemical inhibitors, nystatin and progesterone, which are known to disrupt caveolae or lipid rafts. However, the mixture of these inhibitors did not affect CV-A9 infectivity (**II / Fig. 1A)**. Instead, overexpression of dominant-negative caveolin-3 significantly inhibited infection, which was contradictory to the inhibitory results and to the siRNA screen where the silencing of caveolin-1 (**II / Fig. 3A**) did not reduce virus infection. To clarify the role of caveolae in CV-A9 endocytosis, a caveolin-1-silenced cell line, A549-C9, was generated by using a retroviral RNAi vector. As a result CV-A9 was endocytosed as efficiently into the silenced cell line as into the control cells (A549-RVH1; **II / Fig. 6C**,). To rule out the possibility that residual expression of caveolin-1 mediates the infection in silenced cells, HuH7 cells shown to be devoid of caveolae (Vainio et al., 2002) were also used. Infection of CV-A9 was efficient in HuH7 cells (**II / Fig. 6D**) confirming that caveolin-1 does not mediate the endocytosis of CV-A9 and thus the caveolin-1-mediated route is not important in the CV-A9 infection cycle.

#### 5.2.1.4 Coxsackievirus A9 internalization is mediated by β2-microglobulin, Arf6 and dynamin

Although the endocytosis of CV-A9 has previously been shown to be dependent on β2-microglobulin (β2M; Triantafilou et al., 1999), the exact role of β2M in virus infection has remained unclear. To verify the importance of β2M in the postattachment stage of virus internalization, the protein was silenced by transfecting A549 cells with siRNAs and following CV-A9 entry at time points 0 min, 5 min, 20 min and 6 h (**II / Fig. 8**). In control cells, CV-A9 started to enter the cytoplasm after 5 min while the internalization was arrested in silenced cells and virus particles still remained on the cell periphery at 20 min postinfection. However, after one hour CV-A9 endocytosis continued in the β2M-silenced cells but this did not result in the production of new virus progeny (data not shown) thus suggesting that CV-A9 is directed to an unproductive endocytic pathway in the absence of β2M. These data indicate that β2M is essential in the early stages of CV-A9 entry possibly controlling the vesicular transport.

Since Arf6 (ADP-ribosylation factor 6) has recently been shown to participate in the entry of baculoviruses (Laakkonen et al., 2009), in unproductive internalization of coxsackievirus B3 (CV-B3; Marchant et al., 2009) and in the internalization of major histocompatibility class I (MHC-I) proteins (Naslavsky et al., 2003, Brown et al., 2001), the involvement of Arf6 in CV-A9 endocytosis was also studied. Proliferation of CV-A9 was measured in cells transfected with two individual Arf6 siRNAs and the result showed that one of them inhibited the infection while the other one had no effect (**II / Fig. 9A**). A549 cells were also transfected with plasmid constructs overexpressing wild type (wt) or dominant-negative (DN) forms of Arf6. As a result, virus infection was significantly decreased in the cells expressing DN-Arf6 (**II / Fig. 9B)**. Finally, colocalization analysis between Arf6 and CV-A9 was performed (**II / Fig. 9C**). At 5 min postinfection, Arf6 staining pattern was similar to CV-A9 suggesting that they were in a close proximity and probably in the same structures although strong colocalization was not detected (9%).

The data suggest that in the RGD-dependent cell model Arf6 participates in CV-A9 internalization at least in the early stage of entry. These results have been confirmed by the observation that Arf6 colocalizes with CV-A9 on A549 cell surface and inside the cell until 30 min after CV-A9 infection (unpublished data).

Dynamin was originally thought to participate in clathrin-mediated endocytosis only, but it has recently been shown to be important in other endocytic pathways as well (Doherty and McMahon, 2009). Since dynamin 2 has been shown to be required for the internalization of numerous viruses, its role in CV-A9 entry was analyzed. When the infection was followed in the A549 cells overexpressing the DN form of dynamin 2, the efficiency of infection was reduced by 50% compared with the control cells (**II / Fig. 7A**). However, in an siRNA panel (**II / Fig. 3A and Table 3 in the Materials and methods**) no reduction in infectivity was detected by using two individual siRNAs against dynamin 2. Because the silencing efficiency was not complete (80%), it was possible that the remaining 20% of dynamin 2 expression is sufficient to promote virus entry. The role of dynamin was therefore further studied by dynasore that is a cell-permeable, noncompetitive dynamin GTPase activity inhibitor. Dynasore effectively blocked the virus infection and caused CV-A9 accumulation on the cell periphery suggesting that dynamin is essential for CV-A9 endocytosis (**II / Fig. 7B)**.

### 5.2.2 RGD-independent entry (III)

#### 5.2.2.1 HSPA5 and β2-microglobulin are central mediators of CV-A9 infection

To confirm the importance of HSPA5 and β2M in the early stages of RGD-independent coxsackievirus A9 infection cycle in SW480 cell line, colocalization study with the virus and β2M or HSPA5 was performed. The expression levels of both proteins were first analyzed on the SW480 cell surface (non-permeabilized cell) and inside the cell (permeabilized cell) in the absence of the virus (**III / Fig. 5A)**. The data showed that β2M and HSPA5 are highly expressed in both locations and they reside in the same structures, colocalizations being 50% on the cell surface and 40% inside the cell. This result supports the previous finding by Triantafilou et al. (2001) that HSPA5 and β2M accumulate in the same compartment on the cell surface. While β2M is very abundant in SW480 cells it was difficult to demonstrate the portion of virus particles that were located in the same structures with β2M at 0 min (4% colocalization) or 5 min (10% colocalization; **III / Fig. 5B**). However, at the later time points (at 15 min and 30 min postinfection), CV-A9 and β2M resided clearly in different subcellular compartments (data not shown). When CV-A9 and HSPA5 were double-stained (**III / Fig. 5C**), they were seen to accumulate and colocalize (29%) in visible spots on the cell surface (0 min). In addition, the pattern of HSPA5 was drastically changed from the pattern observed in uninfected cells (**III / Fig. 5A**), which could be explained either by CV-A9 binding to HSPA5, which prevents the antibody binding or alternatively, the incubation on ice with the virus changed the profile of HSPA5. However, after 5 min postinfection, the virus and HSPA5 were clearly in the

same structures (40% colocalization) in the internal side of the plasma membrane implying that HSPA5 binds CV-A9 on the SW480 cell surface independently of integrins or RGD-motif and penetrates the cell plasma membrane in close contact with the virus.

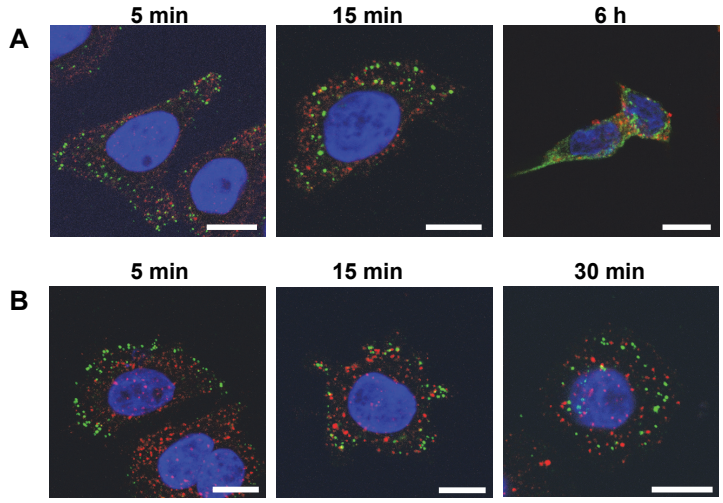


**Figure 10**. CV-A9 does not utilize the clathrin-mediated pathway in SW480 cells. CV-A9 is shown in green and transferrin (A) or EEA1 (B) in red. Time points are shown above the images. Bar 10 µm.

### 5.2.2.2 Caveolin and clathrin routes do not mediate coxsackievirus A9 infection in SW480 cells

Since SW480 cells have been shown to lack caveolin-1 (Berryman et al., 2005), it is unlikely that the caveolin-dependent route plays a role in RGD-independent CV-A9 endocytosis. In addition, the lack of involvement of caveolin route in CV-A9 infection was further supported by the observation that the chemical inhibitor filipin known to inhibit specifically the raft/caveolin-mediated endocytosis (data not shown) did not block viral infection. The role of clathrin-mediated endocytosis (CME) in infection was studied in two separate experiments. Firstly, proliferation of CV-A9 was followed in the presence of $NH_4Cl$ that affects endosomal acidification or chlorpromazine that inhibits clathrin-dependent endocytosis and secondly, the internalization of CV-A9 was followed simultaneously with the classical markers of the clathrin route, transferrin (**Figure 10A**) or endosomal antigen-1 (EEA1; **Figure 10B**). Although significant reduction in virus proliferation was obtained with $NH_4Cl$, the results with chlorpromazine were unlikely to be reliable due to high variation in the infection rates of CV-A9 in separate experiments (data not shown). However, CV-A9 was not observed to internalize in any stage of infection with the classical markers of the clathrin route, which indicated that CME does not have an essential role in CV-A9 infection in SW480 cells.

### 5.2.2.3 Endocytosis occurs via novel Arf6-mediated pathway that is dependent on actin and dynamin

$NA^+/H^+$ exchange inhibitor EIPA (data not shown) and phosphatidyl-inositol 3-kinase (PI3K) inhibitor wortmannin (**III / Fig. 7B**) did not significantly reduce CV-A9 infectivity

in SW480 cell line indicating that viral entry does not occur via macropinocytosis. However, the acting-disrupting agent cytochalasin D inhibited CV-A9 infection almost completely suggesting that actin has a central role in integrin-independent entry. Since the virus infection in A549 cells was shown to be dependent on dynamin, the effect of dynasore was also studied in SW480 cells (**III / Fig. 7**). Dynasore inhibited virus proliferation efficiently indicating that dynamin is essential in both integrin-dependent and integrin-independent endocytosis. While in A549 cells Arf6 was shown to affect virus infection, the significance of Arf6 was also analyzed in SW480 cells. Because the silencing efficiencies of siRNAs against Arf6 were not 100% complete (data not shown), colocalization assays with CV-A9 and Arf6 were performed (**III / Fig. 6 and Figure 11A**). Surprisingly, Arf6 was seen in the same structures with the portion of CV-A9 particles on a cell surface (0 min), colocalization being 11%. In addition, CV-A9 and Arf6 resided in the same vesicles until 30 min after CV-A9 infection (colocalization 55%) implying that Arf6 and CV-A9 are pinched off the plasma membrane at the same site and the endocytosis occurs in the same vesicles perhaps until the viral genome is released. Interestingly, in the parallel experiment with A549 cells, the same result was observed (colocalizations were 11% on the cell surface and 45% after 30 min of the viral infection) confirming that CV-A9 endocytosis is mediated by Arf6 irrespective of the integrins or used cell lines (**Figure 11B**; unpublished data).

## 5.3 Rapid one-step mutagenesis, *in vivo* transcription and expression of GFP from CV-A9 cDNA (IV)

To understand virus behavior, a genetic modification of the viral genome is often essential. Ability to produce proteins such as eGFP would allow real-time monitoring of virus infection, which would be useful to monitor virus infection in tissues and mouse models. Since the traditional mutagenesis of viral cDNAs occurs via several subcloning steps, which are time-consuming (Gullberg et al., 2010, Meyer et al., 2004, Hughes et al., 1995), a long PCR method was established to amplify full-length CV-A9. This was used with mutagenic primers to generate a novel cloning site into which a gene encoding green fluorescent protein (GFP) was cloned. VP1-2A junction was chosen as the site for insertional mutagenesis since in the previous study, the insertion into this site was successful with coxsackievirus B3 (Slifka et al., 2001). The primers used in the mutagenesis contained sequences for modified 2A protease cleavage and NotI restriction sites to facilitate the cloning and the release of GFP from the CV-A9 genome (**IV / Fig. 2**). After amplification, the purified product was re-ligated and transformed directly into bacteria for plasmid rescue. The CV-A9ins plasmid possessed a fragment similar in size (10 kb) compared with the cDNA of the parental CV-A9 indicating that insertional mutagenesis was successful (**IV / Fig. 3)**. Finally, the CV-A9ins plasmid bearing novel NotI restriction enzyme site was used as the backbone vector to subclone a visual marker gene, eGFP. Virus particles were generated in the target cells expressing functional T7 RNA polymerase from cDNA clone containing full-length CV-A9 genome under

control of T7 promoter. A short 120 bp long PCR fragment was identified from cDNA-transfected cells indicating that CV-A9 had multiplied after *in vivo* transcription (**IV / Fig. 1**). In addition, cytopathic effect was detected by visual inspection (data not shown). GFP fluorescence, which indicated virus replication, was visible at 6-8 h postinfection (**IV / Fig. 4**) demonstrating that the developed method is functional for generating viable and modified CV-A9 particles.

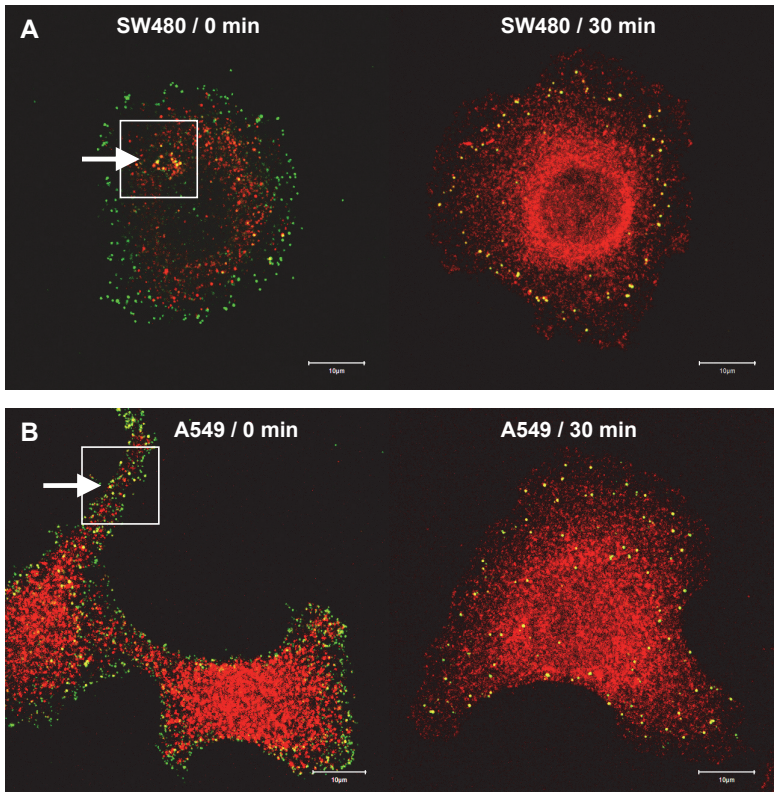


**Figure 11.** Coxsackievirus A9 colocalization with Arf6 in SW480 (A) and A549 (B) cells at 0 min and 30 min postinfection. CV-A9 is shown in green, Arf6 in red and colocalization is visualized in yellow. Squares at 0 min time points highlight the areas containing several colocalization spots and the arrows indicate a single yellow spot. After 30 min of virus infection, Arf6 and CV-A9 are detected in the same vesicles throughout the cells, and they colocalize perfectly. Scale bars are 10 µm and the images are representative slices from the stack images.

# 6 DISCUSSION

The purpose of this thesis was to analyze how coxsackievirus A9 (CV-A9) binds onto the cell surface and internalizes into the cell. The aim was also to develop methods to facilitate real-time imaging of CV-A9 or picornaviruses in general. Although the CV-A9 genome and particle structure have been resolved, the previous results on binding and internalization properties of the virus have been partially contradicting and the endocytosis mechanism has remained unclear. Based on the results of this thesis, αVβ6 is a high-affinity RGD-binding receptor for CV-A9 but the interaction with αVβ6 alone is not sufficient for virus infection. Instead, HSPA5 protein serves as a cellular coreceptor for CV-A9 together with integrin binding. In addition, the penetration of CV-A9 is mediated by HSPA5 protein even in the absence of integrins (i.e. in RGD-independent cell line). Irrespective of the RGD-motif or integrins, CV-A9 penetrates the plasma membrane within novel Arf6-containing vesicles and the early stages of internalization require dynamin, β2-microglobulin and at least to some extent actin. Furthermore, a time-saving method was designed in order to generate GFP-expressing CV-A9 particles by using long PCR and *in vivo* transcription.

## 6.1 Attachment and cell surface interactions of coxsackievirus A9

Previously, it has been demonstrated that coxsackievirus A9 binds to integrins αVβ3 and αVβ6 in various cell models via RGD-motif that resides in the viral VP1 capsid protein (Williams et al., 2004, Triantafilou and Triantafilou, 2003, Triantafilou et al., 1999, Roivainen et al., 1994 and 1991). However, Triantafilou et al. (2000) showed that RGD-less CV-A9 mutant was also capable of interacting with αVβ3 integrin. In addition, CV-A9 has been shown to enter some cell lines, such as RD, independently of RGD-motif suggesting that the virus can utilize both RGD-dependent and RGD-independent internalization (Roivainen et al., 1996, Hughes et al., 1995). Because the first step of the virus infection cycle is virus attachment to cell surface receptors, which determines the tissue tropism and pathogenesis, the role of RGD-motif and integrins in CV-A9 cell surface interactions were further elucidated.

The comparative study with αVβ3 and αVβ6 integrins indicated that αVβ6 integrin serves as a high-affinity RGD-binding receptor for CV-A9. Similar results have been observed with foot-and-mouth disease virus (FMDV) that has been shown to use several αV-integrins, including αVβ6 and αVβ3, for binding *in vitro* (Jackson et al., 2004, 2002, 2000, Duque et al., 2004). However, Monaghan et al. (2005) showed that αVβ6 integrins are constitutively expressed at high levels on epithelial cells that are the targets for FMDV in cattle implying that integrin αVβ6 serves as the primary attachment receptor for FMDV in the natural infection rather than αVβ3, which is mainly expressed in endothelial cells (Singh et al., 2001 and 2000, Breuss et al., 1995 and 1993, Mette et al.,

1993). The epithelial cells are also primary targets for CV-A9 in the human infections, which may both explain the differences in the binding efficiencies between the αVβ3 and αVβ6 integrins and indicate that the role of αVβ6 is probably to direct the CV-A9 particles to specific target cells during the natural infection.

Because it has been shown that FMDV internalizes with αVβ6 receptor via the clathrin dependent entry pathway, the colocalization study of CV-A9 and αVβ6 integrin on RGD- and integrin-dependent A549 cells was performed. Surprisingly, CV-A9 was not observed to colocalize with αVβ6 on the cell surface or internalize with the integrin, which contradict the results of echovirus 1 (E-1) and FMDV: E-1 generates clusters and internalizes into the cell in association with its receptor integrin α2β1 (Marjomäki et al., 2002) and FMDV colocalizes with integrin αVβ6 on the cell surface and inside the cell at 5 min postinfection (Berryman et al., 2005). It should be noted that Berryman et al. used SW480 cell line in which integrin αVβ6 was overexpressed. Thus, this may not represent native infection. In addition, integrin-linked signaling was not required for CV-A9 entry suggesting that αVβ6 integrin serves as an attachment factor for CV-A9 concentrating the virus particles on the cell surface while other receptors are essential for virus internalization. This observation is consistent with the results of Shakeel et al. (2013) who demonstrated by surface plasmon resonance and electron cryo-microscopy that αVβ6 integrin binds to CV-A9 capsid with nanomolar affinity but the interaction between integrin and virion does not induce uncoating. This suggests that RNA release does not occur via integrin receptor binding and hence supports the requirement for other receptors in the RGD-dependent CV-A9 attachment, entry and uncoating.

Since RGD- and integrin-independent CV-A9 attachment occurred in RD and SW480 cells, novel receptors responsible for successful CV-A9 infection were searched using siRNA and peptide phage display library screening. Using these methods two possible candidates were identified: β2-microglobulin (β2M) that is a subunit of major histocompatibility complex class I (MHC-I) complex and heat shock 70kDa protein 5 (HSPA5 protein, also known as BiP or glucose-regulated protein 78kDa, GRP78) that is best known as an ER luminal protein but is also found on the plasma membrane (Reddy et al., 2003, Delpino and Castelli, 2002). Both molecules have been described to cooperate with integrins but this is the first time when they participate in CV-A9 infection independent of integrin molecules. However, direct binding between the virus and β2M was not detected suggesting that the attachment on the cell surface does not occur via β2M despite their close association, which has also been reported previously by Triantafilou et al. (1999). The peptide ESPLSLVA that was found in a phage library screening was identical to the N-terminal sequence of HSPA5 protein and completely blocked virus infection in both SW480 and A549 cells suggesting that regardless of the used cell lines, CV-A9 interacts with the N-terminal region of HSPA5. In addition, HSPA5 was observed to colocalize with CV-A9 on SW480 cell surface, which confirmed the involvement of HSPA5 in CV-A9 binding and was consistent with the previously reported finding by Triantafilou et al. (2002). However, Triantafilou et al. (2002)

suggested that HSPA5 serves as a coreceptor for CV-A9 together with integrins whereas in this study HSPA5 was observed to serve as a primary attachment receptor for the virus in integrin-independent interaction.

Jindadamrongwech et al. (2004) showed that antibodies directed against the N-terminus of plasma membrane bound HSPA5 protein inhibit the binding and infection of dengue virus serotype 2 while the antibodies directed against the C-terminus of the protein enhance the infection but do not affect binding. This implies that antibody binding to the C-terminus probably induces conformational changes in the HSPA5 protein, which then leads to viral uptake. HSPA5 has also been found to serve as a cell binding receptor for Borna disease virus (Honda et al., 2009) indicating that HSPA5 might have a significant role in the natural infection cycle of the viruses. Furthermore, HSPA5 has been shown to be involved in the folding and assembly of MHC class I (Paulsson et al., 2001), which explains the close association of HSPA5 and β2M on the cell surface (Triantafilou et al. 2001). Thus, these observations may explain why both β2M and HSPA5 are essential in CV-A9 binding and internalization.

In a recent study, membrane associated heparan sulfate/heparin class of proteoglycans (HSPG) has been suggested to be a possible (co)receptor for some CV-A9 strains (McLeish et al., 2012). However, according to McLeish et al. (2012), Griggs strain of CV-A9 that was used in this thesis does not bind heparan sulfate (HS) without introduction of a single amino acid mutation (T→R) into the position of 132 in the VP1 protein (T132R), which contradicts the unpublished data by Karelehto et al. (Karelehto, Merilahti, Himanen and Susi, 2013; manuscript in preparation). They used CV-A9 (Griggs strain) and demonstrated that virus infection is affected by interference of HS biochemical pathway and blocked by heparin, protamine and heparinase treatment. They also demonstrated that HS competes with integrin αVβ6 binding *in vitro*. Finally, they show that fifty clinical CV-A9 isolates have similar VP1 sequence as CV-A9 Griggs. Collectively, these data suggest that most CV-A9 isolates bind HS and there is no specific HS-binding site as suggested by McLeish et al. (2012). Nevertheless, the role of HS in natural CV-A9 infection remains to be elucidated.

## 6.2 Internalization of coxsackievirus A9

Studying virus endocytosis has become ever more complex. Modern techniques such as live cell and confocal imaging have helped to identify various new endocytic pathways besides clathrin- and caveolin-mediated endocytosis and macropinocytosis. Most viruses use endocytosis for delivering their genome into the cell because this mechanism does not leave traces of the viral presence on the plasma membrane and therefore causes a delay of the immune response. From the numerous cellular endocytic routes, viruses have evolved to follow a particular pathway which may be determined by particle size, primary target tissues or pH dependence. Nevertheless, a single purpose of viruses is to safely transfer their genome into the cell for initiating virus replication.

In this work αVβ6 integrin was not detected to deliver CV-A9 into A549 cells. In addition, integrins were not observed to facilitate virus attachment on SW480 cells indicating that another receptor is capable of inducing CV-A9 endocytosis. Since HSPA5 was shown to interact with CV-A9, their simultaneous internalizations were followed in SW480 cells in order to demonstrate whether HSPA5 mediates CV-A9 penetration. Interestingly, CV-A9 particles and HSPA5 proteins were seen in the same structures 5 min after virus infection suggesting that HSPA5 and CV-A9 accumulate within the same lipid compartment on a cell surface and penetrate the plasma membrane together. This is the first demonstration showing that HSPA5 protein serves both as an attachment receptor and mediator of CV-A9 infection irrespective of integrins or viral RGD-motif. Furthermore, after 15 min of infection HSPA5 and CV-A9 resided in separate compartments suggesting that they are probably sorted into different endosomal vesicles within 5-15 min after infection. Perhaps HSPA5 is transported back to the plasma membrane within recycling endosomes or through the classical endosomal pathway for degradation whereas CV-A9 is sorted into the endosome where the particle uncoating and genome release occur. However, in contrast to αVβ6 integrin (Monaghan et al., 2005), HSPA5 protein has been found to be present in all cell types (Henderson, 2004, Kiang and Tsokos, 1998) and could therefore be a universal way of the cell to bind various ligands on the cell surface and mediate the specific signals into the cell or to the surrounding environment.

Although β2-microglobulin did not bind directly to CV-A9 particle, its involvement in CV-A9 internalization is evident. In addition, β2M has been suggested to be essential for echovirus 1, 7, 11 and 25, because their proliferation can be prevented by antibody against β2M (Marjomäki et al., 2002, Ward et al., 1998). To study the role of β2M in the postattachment stage of CV-A9 infection, cointernalization of β2M and CV-A9 was followed in A549 and SW480 cells. However, there were no signs of colocalization. Furthermore, in β2M-depleted A549 cells infected with CV-A9 virus internalization was first arrested and then after 1 h of infection, particles started to enter the cell through an unproductive pathway. Ward et al. (1998) showed that β2M blocks echovirus infection in a postattachment stage but prior to RNA translation and replication indicating that β2M might have a role in echovirus uncoating. This may also be the case with CV-A9 albeit a detailed mechanism of β2M in virus infection cycle needs further studies.

In order to identify the endocytic pathway utilized by CV-A9, the involvement of classical clathrin-mediated pathway, caveolin/raft-dependent route and macropinocytosis were examined. Collectively, the results indicated that CV-A9 does not use any of these entry routes although the chemicals that inhibit the actin dynamics partially reduced the infection in A549 cells (jasplakinolide) and almost completely blocked the endocytosis of CV-A9 in SW480 cells (cytochalasin D) suggesting the involvement of macropinocytosis in the CV-A9 endocytosis (Mercer and Helenius, 2012 and 2009). Despite the strict link of actin to macropinocytosis, the actin cytoskeleton may be involved in membrane invagination, coated pit formation, coated pit sequestration, detachment of the newly formed vesicle and the movement of the endocytic vesicles away from the plasma membrane into

the cytosol. Thus the involvement of actin is not specific to any endocytic pathway (Qualmann et al., 2000). Furthermore, the role of cholesterol in CV-A9 endocytosis was partially ignored because cholesterol is involved in various cell membrane functions and therefore it is difficult to postulate the reason for the resulting inhibitory effect of drugs influencing cholesterol albeit cholesterol is generally linked to the clathrin- and caveolin/raft-dependent routes and macropinocytosis (Mercer et al., 2010).

The small GTPase Arf6 (ADP-ribosylation factor 6) that regulates membrane trafficking pathways and structural organization of the cell (D'Souza-Schorey and Chavrier, 2006) was found to have a crucial role in CV-A9 internalization. Firstly, the silencing of Arf6 and the overexpression of DN Arf6 resulted in a clear decrease in the infection in A549 cells and secondly, Arf6 was seen to colocalize with CV-A9 on A549 cell surface and inside the cell (unpublished data). In addition, colocalization in SW480 cells was identical to A549 cells implying that Arf6 containing vesicles deliver CV-A9 into the cell cytosol irrespective of the used cell line. These findings are consistent with the results of Naslavsky et al. (2004 and 2003) who demonstrated the existence of early endosomes that are independent of clathrin but contain β2-microglobulin and Arf6. In addition, Naslavsky et al. (2003) showed that one portion of Arf6-positive endosomes fuses with EEA1-, transferrin-, and Rab5-containing early endosomes that mature to late endosomes and lysosomes, while the other portion of endosomes recycles back to the plasma membrane. These Arf6-associated tubular recycling endosomes contain MHC-I but not clathrin cargo proteins. Interestingly, the recycling to the plasma membrane can be fast occurring directly from the sorting endosome or slow occurring through a pericentriolar endosomal recycling compartment (ERC; reviewed by Schweitzer et al., 2011, D´Souza-Schorey and Chavrier, 2006 and Maxfield and McGraw, 2004) and the longer-lived ERCs consist of a heterogenous subset of endosomal population, which may vary among cell types and thus explains partially the difficulties of identifying these vesicles. Perhaps these endosomes are essential organelles for CV-A9 uncoating since Arf6 and CV-A9 were seen to colocalize even after 30 min postinfection.

Although the Arf6-mediated pathway is thought to occur without the large GTPase dynamin that serves as a scission factor for pinching of the vesicles from the plasma membrane (Mayor and Pagano, 2007), the results of this work suggest that CV-A9 endocytosis is dynamin-dependent: dynasore, which is a cell-permeable, noncompetitive dynamin GTPase activity inhibitor, blocked virus infection and caused CV-A9 accumulation on the cell periphery in both A549 and SW480 cell lines. In addition, the effect of the drug was already observed at 0 min postinfection, which implies that the function of dynamin is essential for CV-A9 penetration. Interestingly, herpes simplex virus structural protein VP22 and baculovirus have also been reported to use dynamin-dependent Arf6-regulated endocytosis (Laakkonen et al., 2009, Nishi and Saigo, 2007). While the entry of baculovirus resembles phagocytosis because it is regulated by dynamin, Arf6 and RhoA (Mercer et al., 2010, Laakkonen et al., 2009), the endocytosis of VP22 and CV-A9 occurred independently of RhoA activity. To our knowledge, this is

the first demonstration of picornaviruses using an Arf6-mediated cellular pathway that is dependent on dynamin and to some extent actin but independent of regulatory factor PI3K.

## 6.3 Rapid one-step mutagenesis of CV-A9 genome and generation of viable GFP-expressing virus particles

Viruses are useful devices in gene therapy and in carrying macromolecules and drugs into the cell because of their capabilities of entering the cell through several cellular pathways and captivating the cellular machinery for their own purposes. Viruses are also potential tools for a targeted elimination of cancer cells and important model systems in molecular, structural and cell biology. In addition, to understand the viral functions themselves, the genetic modification of the viral genome is often essential. Four different strategies have been used to construct recombinant picornaviruses. Foreign sequences can be inserted within the open reading frame (ORF) of the poliovirus capsid proteins or an additional internal ribosome entry site driving a second ORF for the foreign protein expression can be added into the host genome. Furthermore, structural protein sequences can be replaced with foreign genes after which the genome is packaged into the infectious virus in the proper cell line and finally, the foreign gene can be inserted in frame with the viral polyprotein followed by an artificial protease cleavage site (Alexander et al., 1994, Andino et al., 1994, Choi et al., 1991, Burke et al., 1988). The first three methods, however, have restrictions in insert size or the resulting viruses are unstable and defective whereas the last strategy exploits the viral proteases permitting the foreign protein to be cleaved from the viral polyprotein and thus allowing greater diversity in the size of the inserted gene. Although polioviruses are predominantly used as recombinant enteroviruses, Höfling et al. (2000) have demonstrated that these strategies can be applied to an attenuated coxsackievirus as well.

Because the manipulation of the enteroviral genome and the production of the modified viral particles include several time-consuming and inefficient steps, a rapid time-saving method for generating modified, GFP-expressing coxsackievirus A9 particles was designed. The aim was first to demonstrate that the virus particles can be generated from cDNA clones driven by T7 promoter or from full-length PCR products with T7 promoter in target cells that express functional T7 RNA polymerase (T7RNApol). Second, the aim was to amplify and mutate the full-length CV-A9 cDNA clone in a single step and third, the functionalities of the used methods were verified by subcloning a gene encoding green fluorescent protein (GFP) into the VP1-2A junction of the CV-A9 cDNA backbone followed by *in vivo* transcription. VP1-2A junction was chosen as the target for the insertional mutagenesis since Slifka et al. (2001) have successfully used the same insertion site for the mutagenesis of coxsackievirus B3. These steps together allow a rapid delivery of mutagenized viral genomes into host cells for virus production or protein expression.

Several enteroviral cDNAs including CV-A9, which contained the T7 promoter, were successfully transcribed into infectious viruses *in vivo* by using a T7 RNA polymerase -mediated transcription method where the T7RNApol construct was transfected into target cells a day prior to viral constructs. Secondly, two back-to-back primers for introducing the modified 2A protease cleavage and the unique NotI restriction sites into the cDNA of CV-A9 (Hughes et al., 1995) were used in a long PCR reaction with the Phusion™ DNA polymerase. In addition, the PCR reaction was performed without a tedious optimization process which was possible due to the exceptional properties of Phusion (Thermo Fisher, Lithuania). The functionality of the amplified genome was further characterized by introducing GFP in the NotI site of the CV-A9ins plasmid and fluorescence from CV-A9-GFP was detected. The growth of modified particles was similar to the native virus (personal observation) implying that the designed strategy for generating viable and modified coxsackievirus A9 particles by using *in vivo* transfection was successful. Similarly to the study by Lim et al. (2005) who demonstrated that cardiotropic CV-B3 can be used to express GFP in the heart, GFP-expressing CV-A9 particles could be used as a tool for analyzing the progression and spreading of CV-A9 infection in tissue cultures or in mouse models, which would give further information about the infection mechanisms of coxsackievirus A9.

# 7 CONCLUSION

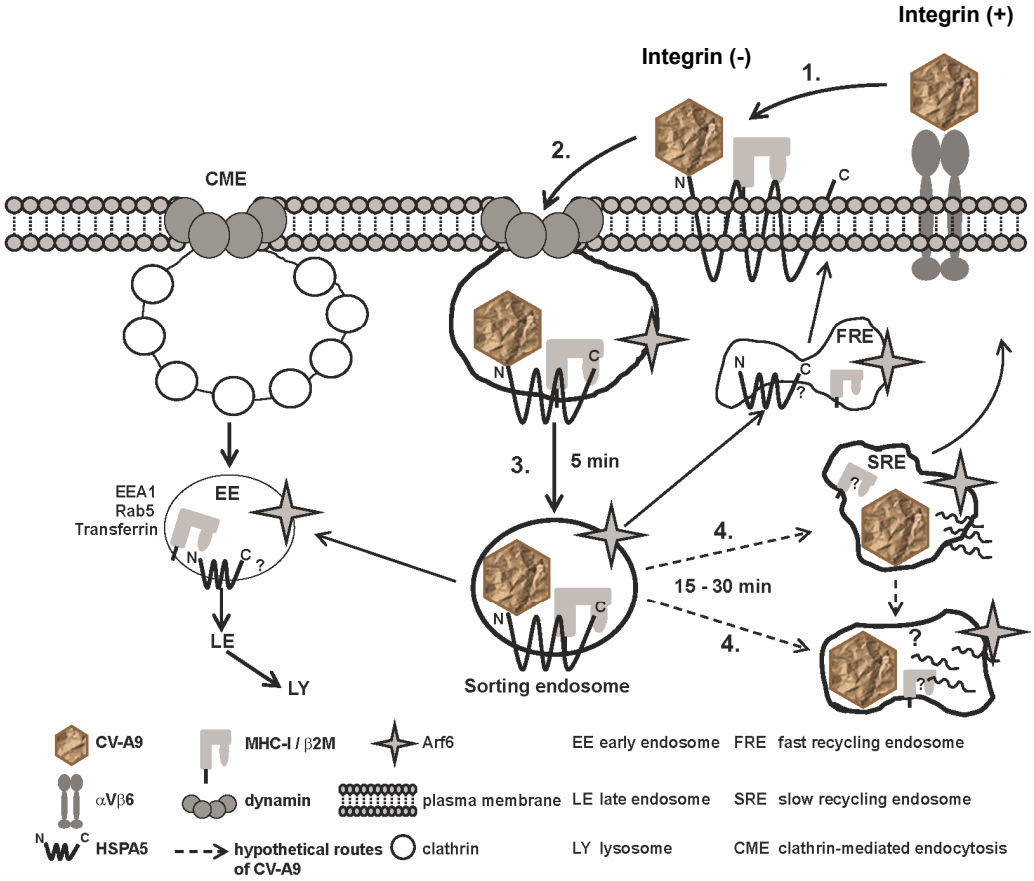


**Figure 12**. The endocytosis of coxsackievirus A9. **(1.)** On the cell surface, CV-A9 attaches to the N-terminus of HSPA5 or alternatively first with αVβ6 integrin and then with HSPA5 protein after which viral penetration **(2.)** occurs within the Arf6 containing invagination together with HSPA5. **(3.)** The formed vesicle is pinched off the plasma membrane with the help of dynamin and at 5 min postinfection, CV-A9 resides in the sorting endosome containing HSPA5, Arf6 and MHC-I including β2-microglobulin (β2M). The sorting endosome can fuse with the EEA1-, Rab5- and transferrin-containing early endosome that follows the classical endocytic pathway. In addition, the sorting endosome can recycle back to the plasma membrane by using fast (FRE) or slow (SRE) recycling routes. **(4.)** Since CV-A9 colocalizes with Arf6 still after 30 min of infection, it probably stays in the SRE or in another, still unknown Arf6-containing structure until the viral uncoating occurs. At 15 min postinfection, CV-A9 and HSPA5 are in different compartments implying that HSPA5 may recycle back to the plasma membrane or it may be targeted into the classical endocytic pathway for degradation. Integrin (+) indicates integrin and RGD-dependent attachment and Integrin (-) integrin and RGD-independent binding.

In conclusion, a model for coxsackievirus A9 cell-surface attachment and endocytosis is proposed (**Figure 12**). The RGD-dependent attachment on a cell surface occurs via integrin αVβ6, which has a likely role in mediating binding of CV-A9 to epithelial cells

during natural infection. However, αVβ6 does not induce virus penetration. Because the plasma membrane spanning protein HSPA5 was found to bind and mediate viral internalization independently of integrins and RGD-motif and the blocking of HSPA5 prevented CV-A9 infection in RGD-dependent and RGD-independent cell lines, HSPA5 is proposed to mediate viral penetration in both cases. However, after 15 min of infection, HSPA5 and CV-A9 are located in separate endosomal vesicles implying that HSPA5 mediates the first steps of CV-A9 entry. HSPA5 protein has also been shown to interact with MHC-I molecules on a cell surface, which explains the involvement of MHC-I subunit β2-microglobulin in CV-A9 infection although the mechanism is unknown. After the binding, CV-A9 is localized into Arf6-positive invaginations on the plasma membrane in association with HSPA5 and the formed structure is pinched off from the plasma membrane with the support of dynamin. The formed vesicle fuses with early endosome that matures to late endosome and lysosome or it recycles back to the plasma membrane using a fast or a slow recycling mechanism. Since Arf6 was seen to colocalize with CV-A9 30 min postinfection, it is unlikely that CV-A9 utilizes the fast recycling pathway. However, CV-A9 was not seen in the early endosomes of classical pathway, which indicates that CV-A9 resides in the slow recycling Arf6-endosome or in another unknown Arf6-containing structure. Although the exact role of β2M in CV-A9 infection is still unclear, it affects viral endocytosis in a postattachment stage perhaps by participating in virus uncoating, since CV-A9 ends up to an unproductive pathway in β2-microglobulin-depleted cells. Further studies are needed to elucidate the mechanisms of action of β2M and HSPA5 in CV-A9 binding and endocytosis. Furthermore, GFP-expressing CV-A9 may prove to be useful in studies related to virus movement in multicellular organisms.

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