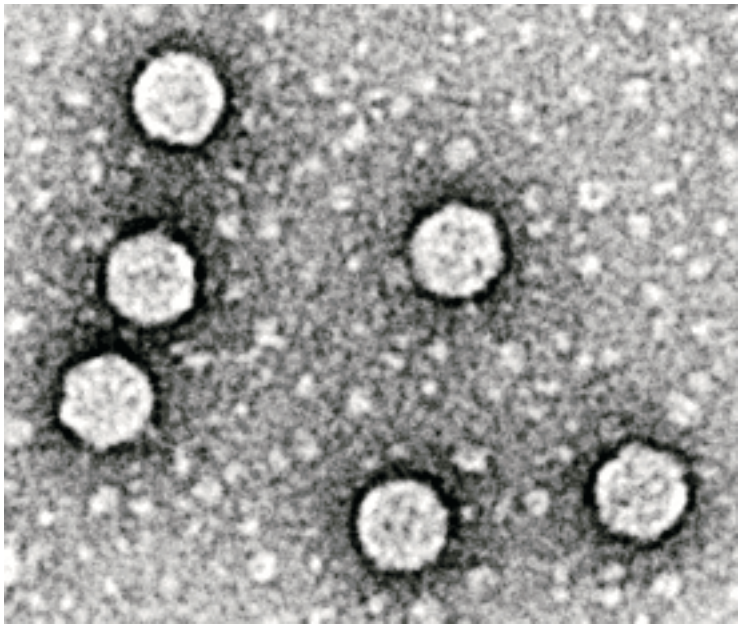
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
189

Paula Upla

Integrin-Mediated Entry of Echovirus 1



JYVÄSKYLÄN YLIOPISTO

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 189

Paula Upla

Integrin-Mediated Entry of Echovirus 1

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi Villa Ranan Blomstedtin salissa
helmikuun 8. päivänä 2008 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
in the building Villa Rana, Blomstedt Hall, on February 8, 2008 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2008

Integrin-Mediated Entry of Echovirus 1

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 189

Paula Upla

Integrin-Mediated Entry of Echovirus 1

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2008

Editors
Varpu Marjomäki
Department of Biological and Environmental Science, University of Jyväskylä
Irene Ylönen, Marja-Leena Tynkkynen
Publishing Unit, University Library of Jyväskylä

Jyväskylä Studies in Biological and Environmental Science

Cover picture by Paula Upla

URN:ISBN:9789513931094
ISBN 978-951-39-3109-4 (PDF)

ISBN 978-951-39-3087-5 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2008

ABSTRACT

Upla, Paula
Integrin-mediated entry of echovirus 1
Jyväskylä: University of Jyväskylä, 2008, 86 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 189)
ISBN 978-951-39-3109-4 (PDF), 978-951-39-3087-5
Yhteenveto: Echovirus 1:n integriinivälitteinen sisäänmeno soluun
Diss.

Echovirus 1 (EV1) is a human pathogen, which belongs to the *Picornaviridae* family of RNA viruses. Here, the life cycle of EV1 was analyzed in detail. Binding of EV1 to the cell surface α2β1 integrin, a collagen receptor, was not accompanied by a conformational change in the virus capsid needed for the release of the RNA genome. EV1 pentamer is supposed to bind five integrin heterodimers at the same time and, behaving as a multivalent ligand, to cause integrin clustering. Clustering of integrins is important for signaling events. Formation of integrin clusters was initiated using primary anti-α2 integrin and secondary antibodies. The receptor clusters were seen to move laterally from glycosyl phosphatidylinositol anchored protein (GPI-AP) enriched domains along cortical actin filaments and to subsequently internalize. The internalization process was dependent on protein kinase Cα (PKCα) activity and other signaling events. Integrin α2β1 clustered by antibodies or EV1 was internalized into perinuclearly accumulated vesicles positive for caveolin-1, but not in endosomal vesicles of the clathrin-dependent entry route.

The perinuclear vesicles were caveosomes, since EV1 partially colocalized there with simian virus 40 (SV40) and cholera toxin, known to traffic via caveosomes. However, colocalization of EV1 with caveolin-1 was not evident at the plasma membrane, but became apparent during the first 10 to 20 min of internalization. EV1 did not move to any other cellular locations from caveosomes. Presumably the EV1 RNA genome is released directly from the caveosomes to initiate replication in the cytoplasm.

The replication of EV1 was dependent on cytosolic Ca^{2+}-activated cysteine proteases, calpains, which degrade a number of cytoskeletal and cytoplasmic proteins. Both calpains 1 and 2 were recruited into caveosomes and they were activated concomitantly with the increase in Ca^{2+} concentration. Calpain inhibitors, as well as siRNAs for calpains 1 and 2 blocked EV1 infection. However, calpain inhibition did not have preventive effect on the *in vitro* translation of viral proteins.

Key words: echovirus 1, integrin, viral entry, caveosome, replication, calpain

Paula Upla, University of Jyväskylä, Nanoscience Center/Department of Biological and Environmental Science, Survontie 9, FI-40014 University of Jyväskylä, Finland

Author´s address

Paula Upla
Nanoscience Center/Department of Biological
and Environmental Science
Survontie 9
FIN-40014 University of Jyväskylä,
Finland

Supervisors

Professor Jyrki Heino, M.D., Ph.D.
Department of Biochemistry and Food Chemistry
Vatselankatu 2
FIN-20014 University of Turku,
Finland

Academy research fellow Varpu Marjomäki, Ph.D.
Nanoscience Center/Department of Biological
and Environmental Science
Survontie 9
FIN-40014 University of Jyväskylä,
Finland

Reviewers

Professor Elina Ikonen, M.D., Ph.D.
Institute of Biomedicine
Haartmaninkatu 8
FIN-00014 University of Helsinki
Finland

Adjunct Professor Eeva-Liisa Eskelinen, Ph.D.
Department of Biological and
Environmental Sciences
Viikinkaari 5 D
FIN-00014 University of Helsinki
Finland

Opponent

Professor Oleg Shupliakov, M.D.
Department of Neuroscience
Karolinska Institutet
Von Eulers vägen 3
SE-17177 Stockholm
Sweden

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following scientific articles and a manuscript, which will be referred to in the text by their Roman numerals.

I Marjomäki, V., Pietiäinen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypiä, T. & Heino, J. 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76: 1856-1865.

II Upla, P., Marjomäki, V., Kankaanpää, P., Ivaska, J., Hyypiä, T., van der Goot, G. & Heino, J. 2004. Clustering induces a lateral redistribution of $\alpha 2\beta 1$ integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol. Biol. Cell. 15: 625-636.

III Pietiäinen, V., Marjomäki, V., Upla, P., Pelkmans, L., Helenius, A. & Hyypiä, T. 2004. Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II and signaling events. Mol. Biol. Cell. 15: 4911-4925.

IV Upla, P., Marjomäki, V., Nissinen, L., Nylund, C., Waris, M., Hyypiä, T. & Heino, J. 2007. Calpain 1 and 2 are required for the replication of echovirus 1. Manuscript.

RESPONSIBILITIES OF PAULA UPLA IN THE ARTICLES AND MANUSCRIPT OF THIS THESIS

Article I:

I am responsible for the immuno-isolation of caveolar structures with anti-caveolin-1 coated magnetic beads and the subsequent SDS-PAGE and Western blot analysis of the isolated material.

Article II:

I participated in the immunofluorescence and electron microscopy studies. I am responsible for the studies of virus and integrin internalization, ERK activation, transient transfections of signaling proteins and participation of PKCα in the infection.

Article III:

I performed the experiments, in which the effects of actin-disturbing agents and mutants of Eps15 and AP180 were studied in CV-1 cells.

Article IV:

I am responsible for the internalization, immunofluorescence and immunoblotting studies, siRNA studies and infections, in which the effects of chemical inhibitors on the number of infected cells were studied. I also performed the electron microscopy, metabolic and *in vitro* translation experiments. I wrote the article with Jyrki Heino, Varpu Marjomäki and Timo Hyypiä.

All the studies were performed under supervision of Jyrki Heino and Varpu Marjomäki.

ABBREVIATIONS

293	human kidney cell line
A549	human lung carcinoma cell line
ARF	ADP ribosylation factor
βTD	β-tail domain
CAR	coxsackie-adenovirus receptor
CAV	coxsackie A virus
CVB	coxsackie B virus
CCP	clathrin coated pit
CRE	*cis*-acting replication element
CV-1	African green monkey kidney cell line
DAF	decay acceleration factor
ECM	extracellular matrix
EEA-1	early endosomal antigen-1
EGF	epidermal growth factor
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
EV1	echovirus 1
FAK	focal adhesion kinase
FISH	fluorescent *in situ* hybridization
FMDV	foot-and-mouth disease virus
GFP	green fluorescent protein
GMK	green monkey kidney cell line
GPI-AP	glycosyl phosphatidylinositol anchored protein
GTPase	guanosine triphosphate hydrolyzing enzyme
HAV	hepatitis A virus
HCMV	human cytomegalovirus
HeLa	cervical carcinoma cell line
HEV	human enterovirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPEV	human parechovirus
HRV	human rhinovirus
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IRES	internal ribosome entry site
LDL-R	low density lipoprotein receptor
LLC-MK	monkey kidney cell line
LV	Ljungan virus
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MIDAS	metal ion-dependent adhesion site
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PEST	proline, glutamic acid, serine, threonine

p.i.	post infection
PI3K	phosphoinositide-3 kinase
PIP2	phosphatidylinositol 4,5 -biphosphate
PKC	protein kinase C
PFU	plaque forming unit
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PSI	plexins, semaphorins, integrins
PTB	polypyrimidine tract binding protein
PV	poliovirus
RGD	arginine-glysine-aspartic acid
RRL	rabbit reticulocyte lysate
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SAOS	human osteosarcoma cell line
SDS	sodium dodecyl sulfate
SNX	sorting nexin
SV40	simian virus 40
TPA	12-O-tetradecanoylphorbol-13-acetate
UTR	untranslated region
VCAM-1	vascular cell adhesion molecule-1
VP	viral protein

1 INTRODUCTION

Picornaviruses have probably existed for most of human history. The oldest evidence for paralytic poliomyelitis is found in Egyptian stone engravings over 3000 years old. Members of the picornavirus family are among the smallest viruses known. The word "pico" stands for very small in Greek. Despite their small size they are important pathogens of both humans and animals. They are responsible for diseases such as poliomyelitis, hepatitis A and foot-and-mouth disease, causing dramatic costs in morbidity, mortality and economic terms. On the other hand, the structural simplicity of the picornaviruses has enabled their use as excellent tools for research on principal cell functions and, especially, RNA replication. The structure and genetics of picornaviruses are well characterized but their pathogenic mechanisms remain unknown. Effective treatment and prevention of diseases requires understanding of the intricate interaction between viral proteins and host cells.

The focus of this doctoral thesis was to study the life cycle of EV1 and the relatively long-lasting interaction of the virus with its receptor, α2β1 integrin. Integrins are the primary sensors of the extracellular environment. They transmit signals bidirectionally across the plasma membrane. Many viruses take advantage of this signaling of integrins in their entry. First, the role of α2β1 integrin in the infection was confirmed and the entry route of EV1 was charaterized (I). Clustering of integrins was used to follow the unique internalization pathway of the virus-receptor complex. Furthermore, signaling proteins in host cells were analyzed to find regulators needed in the process (II). EV1 trafficking was observed by video-enhanced live microscopy with concomitant analyses of signaling events (III) and finally, essential host proteins needed in EV1 replication were found (IV).

A detailed knowledge of viral entry mechanisms elucidates our concept of endocytosis and is helpful for the development of new antivirals. Moreover, understanding of the regulation of integrin trafficking is essential, since integrins as key adhesive receptors, contribute to numerous cellular functions. Aberrations in integrin function can lead to several serious pathological conditions, including thrombosis and cancer.

2 REVIEW OF THE LITERATURE

2.1 Picornaviruses and their classification

Members of the family *Picornaviridae* are among the smallest RNA-containing viruses known. They are widespread in nature and of great medical and agricultural significance. Based on molecular information the picornaviruses are currently classified, not unambiguously though, into nine genera: *Enterovirus, Rhinovirus, Hepatovirus, Parechovirus, Cardiovirus, Aphthovirus, Erbovirus, Kobuvirus* and *Teschovirus* (Stanway et al., 2005) (Table 1). The genus *Enterovirus* consists of important human pathogens, of which poliovirus (PV) is the most extensively studied member. Human rhinoviruses (HRVs) belong to genus *Rhinovirus* and are the causative agents of common cold. Hepatitis A virus (HAV), a member of genus *Hepatovirus*, is the cause of acute hepatitis in humans. Genus *Parechovirus* is characterized by respiratory and gastrointestinal symptoms (Joki-Korpela and Hyypiä, 1998; Tauriainen et al., 2007). It consists of human parechovirus 1 and 2 (HPEV1-2), and recently found serotypes HPEV3-5 (Ito et al., 2004; Al-Sunaidi et al., 2007), together with Ljungan virus (LV), which infects rodents (Stanway and Hyypiä, 1999; Miranti and Brugge, 2002). The genus *Cardiovirus* encompasses two rodent pathogens, and a member of the genus *Aphthovirus*, foot-and-mouth disease virus (FMDV), is responsible for the economically important disease of cloven-foot animals. Aichi virus is a member of genus *Kobuvirus* and associated with oyster-related gastroenteritis. The genera *Erbovirus* and *Teschovirus* include pathogens infecting animals. Every genus is composed of species that are further divided into a wide range of serotypes based on antigenic properties.

TABLE 1 Classification of picornaviruses (Stanway et al., 2005)

Genus	Species (number of serotypes)	Clinical symptoms in man
Enterovirus	**Poliovirus (3)**	Poliomyelitis,
	- Human polioviruses 1-3	gastroenteritis
	Human enterovirus A (12 serotypes)	Meningitis, encephalitis,
	- Human coxsackie viruses A2-A8, A10-A14, A16	paralysis, myocarditis, rash
	- Human enterovirus 71	
	Human enterovirus B (37)	Meningitis, paralysis,
	- Human coxsackieviruses B1-B6	encephalitis, pleurodynia,
	- Human coxsackievirus A9	myocarditis, gastroenteritis,
	- Human echoviruses 1-7, 11-21, 24-27, 29-33	respiratory infections
	- Human enteroviruses 69, 73	
	Human enterovirus C (11)	Respiratory infections,
	- Human coxsackieviruses A1, A11, A13, A15, A17-A22, A24	conjunctivitis
	Human enterovirus D (2)	Conjunctivitis
	- Human enterovirus 68, 70	
	Bovine enterovirus (2)	
	Porcine enterovirus A (1)	
	Porcine enterovirus B (2)	
Rhinovirus	**Human rhinovirus A (74)**	Common cold, otitis
	Human rhinovirus B (25)	Common cold, otitis
Cardiovirus	**Encephalomyocarditis virus (1)**	
	Theiler`s murine encephalomyocarditis virus (2)	
Aphthovirus	**Foot-and-mouth disease virus (7)**	
	Equine rhinitis A virus (1)	
Hepatovirus	**Hepatitis A virus (1)**	Liver disease
	Avian encephalomyelitis virus (1)	
Parechovirus	**Human parechovirus (5)**	Respiratory infections,
	Ljungan virus (2)	gastroenteritis, CNS infections
Erbovirus	**Equine rhinitis B virus (2)**	
Kobuvirus	**Aichi virus (1)**	Gastroenteritis
	Bovine kobuvirus (1)	
Teschovirus	**Porcine teschovirus (10)**	

The genus *Enterovirus* comprises five human enterovirus (HEV) species: HEV-A to HEV-D and poliovirus. Echovirus 1 (EV1) represents the clinically important HEV-B species. Most enterovirus infections cause mild and self-limiting diseases, however, sometimes the illnesses can be severe, even fatal. Poliovirus causing severe poliomyelitis has paralysed or killed millions of people worldwide over the

years, and attempts to eradicate poliovirus have been fruitful but not totally successful so far (Bolten et al., 1998; Thompson and Tebbens, 2007). The other enteroviruses of HEV-B species are frequently associated with diseases such as meningitis, paralysis, encephalitis, myocarditis, gastroenteritis and respiratory infections. Furthermore, viruses of this group have a role in the pathogenesis of type 1 diabetes (Andreoletti et al., 1997; Mäkelä et al., 2006). Enterovirus infections usually start from the respiratory or gastrointestinal mucosa because of typical enterovirus transmission by fecal-oral or respiratory routes. The virus replicates in the mucosa and by spreading through the lymphatics to the circulation finds its way to the secondary replication site, which determines the symptoms of the infection (Roivainen, 2006).

Echoviruses comprise the largest subgroup of enteroviruses. They were initially identified by their property of being cytopathogenic only in cultured cells but not in infant mice, separating them serologically from polioviruses. For this reason and since echoviruses could not be identified as the etiologic agent of a clinically distinct disease, they were given the name "Enteric Cytopathogenic Human Orphan" (Committee on the ECHO viruses, 1955).

2.2 General properties of picornaviruses

2.2.1 Virion structure

Simple and nonenveloped picornaviruses are well characterized biochemically and genetically. They are spherical assemblies of about 30 nm and share similar icosahedral capsid architecture. Crystal structures of many representatives have been determined including EV1 (Filman et al., 1998), PV serotypes (Hogle et al., 1985; Filman et al., 1989; Lentz et al., 1997), several HRVs (Rossmann et al., 1985; Kim et al., 1989; Verdaguer et al., 2000), CVB3 (Muckelbauer et al., 1995), FMDV (Acharya et al., 1989) and CAV9 (Hendry et al., 1999).

The protein shell is made of 60 heteromeric units, protomers, each composed of four capsid proteins: VP1-VP4 (Figure 1). Protomers are arranged in groups of five, called pentamers and 12 pentamers constitute the icosahedral capsid. The structures of VP1, VP2 and VP3 are similar to one another: each folds into an eight-stranded β-barrel with two flanking helices. The cores of the barrels differ in C- and N-terminal extensions and loops that connect the β-strands and give each virus an unique surface structure (Hogle et al., 1985). VP1-VP3 decorate the surface of the virion, whereas VP4 lies hidden within the inner surface of the particle (Hogle et al., 1985). The N-terminus of VP4 in all picornaviruses is covalently linked to a myristic acid group. Upon discovery the myristoylation was suggested to stabilize the virus particle and be involved in virus receptor binding, uncoating and capsid assembly (Chow et al., 1987). This theory has later been supported by studies with site-specific mutants (Moscufo et al., 1993; Danthi et al., 2003; Tosteson et al., 2004). Also, the N-termini of VP1-VP3 are buried in the inner surface of the capsid and form an extensive network that contributes remarkably to the protein shell stability (Hogle et al., 1985).

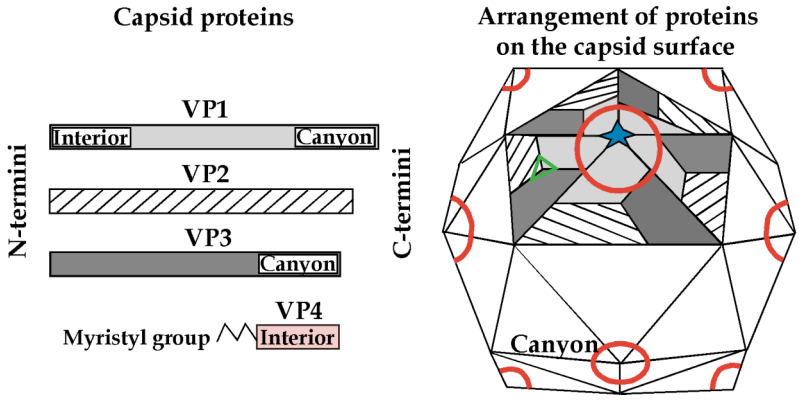


FIGURE 1 Structure of picornaviruses. An icosahedral capsid is composed of 60 copies of proteins VP1-VP4. VP1-VP3 are organized on the outer surface whereas VP4 lies in the interior surface of the capsid. Canyons are indicated with rings, 5-fold symmetry with a star and 3-fold symmetry with a triangle. Modified from Carter and Saunders, 2007.

When the structure of HRV14 was first solved, the presence of deep canyon surrounding every peak of five-fold vertice of the capsid was observed. Residues lining the canyon were found conserved whereas those forming the exposed antigen binding sites were hypervariable (Rossmann et al., 1985; Rossmann, 1989). The canyon floor was suggested to be the receptor binding site since it would exclude the entrance of the bulky antibodies and in this way escape neutralization (Rossmann et al., 1985). Later, several studies have provided proof that PVs, the major group HRVs, CVBs and CAV21 use the canyon base as the receptor attachment site (Colonno et al., 1988; Olson et al., 1993; Bergelson et al., 1997; Kolatkar et al., 1999; Belnap et al., 2000).

Beneath the canyon floor of entero- and rhinoviruses lies a hydrophobic pocket which binds antiviral compounds (Smith et al., 1986). Binding e.g. a fatty acid (pocket factor), to the pocket was thought to stabilize the capsid through competing with binding of the receptor and by having an inhibitory effect on the uncoating (Shepard et al., 1993; Phelps and Post, 1995). A recent study suggests that the pocket itself, not the pocket factor, is crucial in the picornaviral life cycle. The pocket affords flexibility to the capsid which is needed upon receptor binding (Katpally and Smith, 2007).

2.2.2 Genome organization

The capsid proteins of picornaviruses surround a single-stranded, positive sense RNA genome with a length of 7200 to 8500 nucleotides, which contains all the necessary signals for virus replication (Figure 2). The 5´end of the genome is covalently linked to a small basic protein, VPg (Virion Protein genome linked) and a poly(A) tail is present at the 3´end. The open reading frame (ORF) encodes a single, long polyprotein of 247 kDa that is processed by virally encoded proteases into precursor proteins P1, P2 and P3. P1 region contains the four structural proteins (VP1-VP4) and P2 (2A to 2C) together with P3 (3A to 3D) regions contain

the nonstructural proteins required for replication.

ORF is flanked by a long 5´nontranslated region (5´NTR) and a short 3´NTR region. The first segment of the 5´NTR contains a cloverleaf-like domain (Andino et al., 1990; Harris et al., 1994; Xiang et al., 1995) and the rest of the 5´NTR consists of a highly structured internal ribosomal entry site (IRES) (Jang et al., 1988; Pelletier and Sonenberg, 1989). The 5´NTR is the most similar part of the genome, whereas the structures of the 3´NTR are diverse between closely related viruses (Stanway, 1990). The enteroviral 3´NTR folds into two hairpin-loop structures (domains X and Y), in which a poly(A) tail is partially included (Pilipenko et al., 1992a; Mirmomeni et al., 1997). *Cis*-acting replication elements (CREs) that are essential for viral RNA replication, occur at both the 5´ and 3´NTRs (Gerber et al., 2001). An additional CRE is found within the coding region of picornavirus genomes in varying positions (McKnight and Lemon, 1998; Goodfellow et al., 2000; Gerber et al., 2001; Mason et al., 2002).

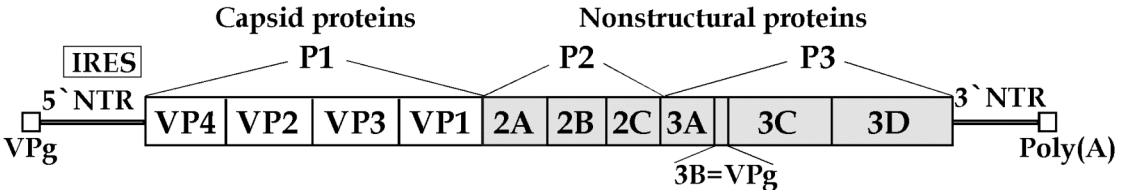


FIGURE 2 Genomic organization of picornaviruses. The genome consists of one ORF flanked by the untranslated regions. The coding region is divided into three portions (P1, P2 and P3), which contain the capsid proteins (VP1-VP4) and the nonstructural proteins (2A-3D) required for replication. Modified from Bedard and Semler, 2004.

2.2.3 Infection cycle

2.2.3.1 Translation and polyprotein processing

To initiate the infection cycle a virus has to bind to cell surface receptors. The receptors may induce conformational changes in the virus capsid leading to internalization and uncoating. Alternatively, they may initiate signaling pathways to promote entry and decapsidation of the genome occurs later intracellularly.
Decapsidated viral RNA is translated to manufacture the virus-coded RNA-synthesizing machinery. Translation is also a prerequisite for replication (Novak and Kirkegaard, 1994). The strategy to replicate via a single polypeptide is unique to picornaviruses. Picornavirus RNA genomes can act directly as mRNAs. They are uncoated in contrast to the cellular mRNAs, which contain 7-methyl guanosine cap structures at their 5´ends (m^7GpppN). The 5´cap structure links eukaryotic mRNAs to the cellular translation machinery. It is recognized by the cap-binding protein eIF4E, which in turn forms a complex with other eukaryotic initiation factors (eIFs). By interacting with 5´end of the mRNA and the eIFs the 40S ribosomal subunit scans the mRNA until it encounters a favourable initiation (AUG) codon. Subsequently it attracts the 60S ribosomal subunit to form a complete translation initiation complex. Picornaviruses translate their RNA genomes by a cap-independent mechanism, which is directed by the IRES (Jang et al., 1988; Pelletier and Sonenberg, 1989). The IRES is composed of very stable and

extensive secondary structures and it enables ribosomes to internally enter the RNA without scanning from the 5´end (Jang et al., 1988; Trono et al., 1988; Pelletier and Sonenberg, 1989). Most of the eukaryotic IFs are involved in the translation.

Picornaviral IRESs are divided into distinct groups on the basis of structural properties (Hellen and Wimmer, 1995). One group involves entero- and rhinoviruses. Translation of PVs and HRVs is inaccurate in rabbit reticulocyte lysate (RRL), unless the lysate is supplemented by HeLa cell extracts (Dorner et al., 1984). The extracts contain cellular RNA-binding proteins (IRES *trans*-acting factors, ITAFs), which stabilize and control the functional state of a particular IRES (Belsham and Sonenberg, 2000). In the past few years an increasing number of cellular mRNAs have also been seen to contain IRES elements associated mostly with control of cell growth and death (Stoneley and Willis, 2004).

Picornavirus infections result in severe shutoff of host cell protein synthesis. This is accompanied by the cleavage of eukaryotic translation initiation factor eIF4G by entero- and rhinovirus encoded protease $2A^{pro}$. Despite inhibition of cellular mRNA translation, picornavirus genomes are efficiently translated (Etchison et al., 1982; Etchison and Fout, 1985). The C-terminal fragment eIF4G, cleaved by $2A^{pro}$, enhances the IRES-mediated translation (Borman et al., 1997). Translation takes place in the endoplasmic reticulum (ER) in a membrane-bound fashion (Bolten and Egger 1998). The nascent picornaviral polyprotein is already cotranslationally processed by at least three viral proteolytic activities. This explains why the full-length viral polyprotein is never seen in infected cells. The primary cleavage is carried out in entero- and rhinoviruses by $2A^{pro}$, which separates the capsid and nonstructural precursor proteins (Toyoda et al., 1986). Processing of the polyprotein produces approximately ten final protein products as well as a number of cleavage intermediates (Figure 3). A relatively small picornaviral RNA genome thus has a powerful coding capacity, since often the intermediate cleavage products can have special functions in the replication cycle, which the final products do not perform. The viral 3CD is a multifunctional precursor of the protease $3C^{pro}$ and the RNA-dependent RNA polymerase $3D^{pol}$. By cleaving P1, 3CD produces the capsid proteins VP0, VP1 and VP3 and the nonstructural proteins 3AB, 3CD, $3C^{pro}$ and $3D^{pol}$. All picornaviruses possess a $3C^{pro}$ proteinase, which is responsible for the majority of the remaining cleavages.

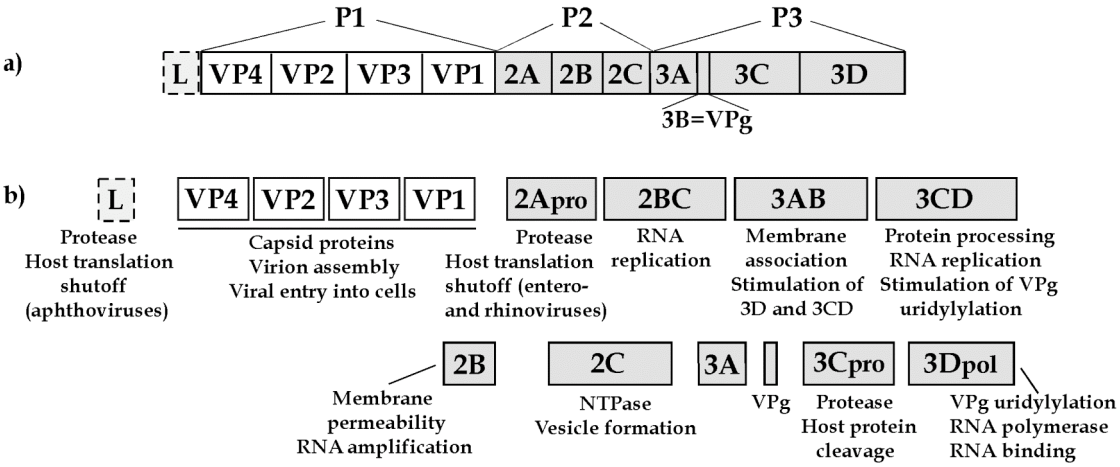


FIGURE 3 Polyprotein processing of picornaviruses. (a) The translated polyprotein is cleaved by viral proteases into precursor proteins P1, P2 and P3. (b) Further cleavages produce intermediate and mature viral proteins. The functions of the proteins during infection cycle are described. Modified from Bedard and Semler, 2004.

2.2.3.2 Replication complex and RNA replication

Our understanding of picornavirus replication is based mainly on studies with PV, and many uncertainties still exist about the details. The first event in the replicative process is copying of the input viral RNA into negative strand RNA, which is then used as a template for the production of copies of positive strand RNAs (Figure 4). Both of the strands are elongated by viral RNA polymerase $3D^{pol}$, which is a primer-dependent enzyme. In uridylylation, which requires interaction between 3CD and $3D^{pol}$ (Pathak et al., 2007), VPg is covalently linked to uridine nucleotides (UMPs) (Paul et al., 1998). The resulting VPgpUpU primes both the positive and negative strand synthesis. The poly(A) tail is used to produce VPgpUpU (Paul et al., 1998). Available evidence supports also the use of CRE within the coding region of picornaviral RNA for uridylylation (Paul et al., 2000; Rieder et al., 2000; Gerber et al., 2001; van Ooij et al., 2006). Whether CRE is required only for priming the positive strand synthesis (Morasco et al., 2003; Murray and Barton, 2003) or for both the positive and negative strand synthesis (van Ooij et al., 2006), is controversial.

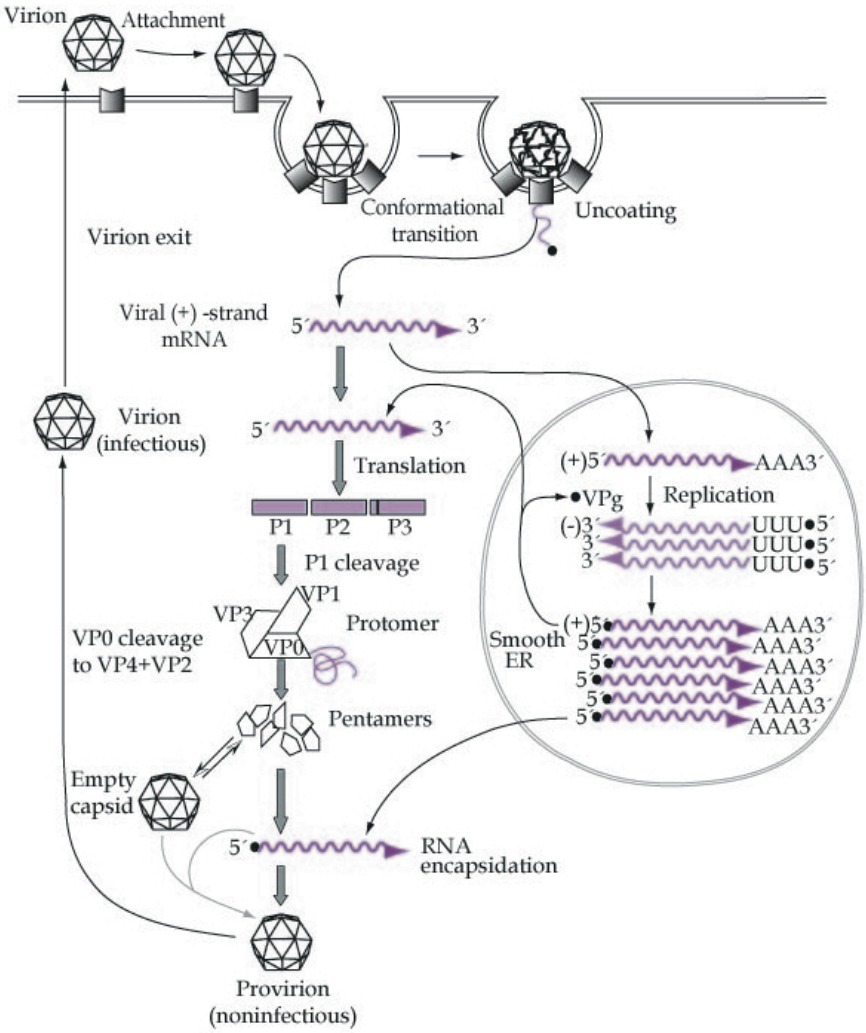


FIGURE 4 Infection cycle of picornaviruses. Binding of virus to receptor is followed by uncoating and genome release into the cytoplasm for replication. Translation produces a polyprotein, which is processed by viral proteases. Replication occurs in association with cellular membranous structures. Assembly of the virus is related to processing of the polyprotein and occurs through a series of intermediates. Lysis of the cells releases the virions. Modified from Hogle, 2002.

To promote negative strand synthesis and repress translation, 3CD binds to the 5´cloverleaf RNA of the viral genome, which contains overlapping signals for translation and replication (Gamarnik and Andino, 1998). Cleavage of cellular polypyrimidine tract binding protein (PTB) by 3CD has also been reported to contribute to the switch from translation to replication (Back et al., 2002). The synthesis of the negative strand is associated with vesicles but is distinct from replication complexes (Bolten et al., 1998). The viral 5´ and 3´ ends interact to form a circular ribonucleoprotein complex to support the start of negative strand synthesis at the 3´end (Barton et al., 2001). The resulting RNA intermediate is double-stranded.

Picornavirus RNA synthesis occurs in replication complexes, which are associated with the cytoplasmic surfaces membranous vesicles (Wimmer et al., 1993; Salonen et al., 2005). Picornavirus infection leads to proliferation and

reorganization of intracellular membranes. Several organelles, such as lysosomes, the Golgi complex and ER contribute to the functional replication complexes (Schlegel et al., 1996). By binding to the viral membrane-associated protein 3AB, $3D^{pol}$ is recuited to the membranes (Lama et al., 1994). CRE within the 3´end of the PV negative strand is needed for efficient template selection and formation of initiation complex for positive strand synthesis (Pilipenko et al., 1992b; Roehl and Semler, 1995; Banerjee et al., 1997). A cellular protein, hnRNPC (heterogenous nuclear ribonucleoprotein C) relocates from nucleus to the cytoplasm (Belov et al., 2000; Gustin and Sarnow, 2001) and binds CRE. Other viral proteins, such as 2C and 3CD, may be recruited to the initiation complex by hnRNPC (Brunner et al., 2005).

Approximately 2500 RNA molecules/min are synthesized in a PV infected cell. More positive than negative strands are generated, since they are used as templates for further negative strand synthesis, mRNAs and genomes of progeny virions. A high evolution rate of picornaviruses derives from the high error rate in replication. RNA polymerase has no proofreading mechanism and it has been estimated that one error/2200 bases takes place (Wells et al., 2001). As a result, a picornavirus genome exists as a large number of variants, termed quasispecies, of which some may enable the virus to adapt to new circumstances.

2.2.3.3 Assembly

The viral 3CD protease cleaves P1 and produces the capsid proteins VP1, VP3 and immature myristoyl-VP0 (Figure 4). The proteins form a pentameric intermediate which spontaneously assembles into an empty capsid (Hogle, 2002). During assembly the uncoating program is built into the virion to make sure that disintegration of the particle occurs upon appropriate cues without external energy. The mechanism of picornavirus RNA genome encapsidation is poorly understood. Nonetheless, since only newly synthesized positive strand RNAs are selectively packaged, viral replication and encapsidation are thought to be functionally linked (Nugent et al., 1999). In one picornavirus, Aichi virus, RNA sequence critical for encapsidation has been identified (Sasaki and Taniguchi, 2003).

The outcome of encapsidation is formation of a precursor, called the provirion. Simultaneously with the encapsidation the myristoyl-VP0 is autocatalytically processed to yield myristoyl-VP4 and VP2. Through maturation cleavage the stability of the virus particle increases significantly (Hogle, 2002).

2.3 Picornavirus-receptor interactions

2.3.1 Common properties in picornavirus-receptor interactions

Viruses can infect only cells, which have appropriate receptors for binding. Distribution of receptors is one determinant of the host and tissue tropisms. Expression of the receptor molecules in a particular tissue partly determines the clinical symptoms that appear. Picornaviruses have evolved to use a wide range of different proteins for entry and they may use different receptors for entry into different cell types (Table 2). The receptor may be the only molecule involved or, alternatively, additional molecules may be needed to complete the entry stage. The virus-receptor interactions may lead to cellular signaling promoting infection and uncoating. Also, they may have an inhibitory effect on the host cell immune response or trigger apoptosis to help virus release.

Binding of PVs, HRVs and related enteroviruses to the receptor is followed by conformational changes in the virus to produce an A particle or 135S particle. The particle sediments at 135S, whereas the native virion sediments at 160S. The receptor-mediated irreversible conversion to the A particle is associated with externalization of myristoyl-VP4 and the N-terminal extension of VP1 (Fricks and Hogle, 1990). The second altered form of the virus is an empty capsid without RNA, sedimenting at 80S. The trigger of this conversion is not known, but it is receptor-independent (Hogle, 2002). Both of the conversions in the virus particle can be induced without receptor by low ionic concentrations and elevated temperatures (Wetz and Kucinski, 1991; Curry et al., 1996). The dependence of uncoating on the elevated temperatures suggests that there is a high activation energy barrier in the conversion pathway (Hogle, 2002). The receptor lowers the activation barrier by acting like a catalyst (Tsang et al., 2001).

The receptor binding site for PVs and for major group HRVs is located in the canyon in contrast to minor group HRVs, and FMDV which have their receptor binding sites exposed at the top of the five-fold axis. Receptor binding in the canyon triggers also the conformational changes in the virus capsid, whereas receptor binding outside the canyon only concentrates viruses on the surface of the cell. Furthermore, as the receptor-binding site locates in a deep invagination, it can provide more surface area for the virus-receptor interaction and thus donate some of its binding energy to facilitate structural alterations (Hogle, 2002).

2.3.2 Cellular receptors for picornaviruses

All the receptors binding to the picornavirus canyon share structural similarities. They are type I transmembrane glycoproteins with two to five extracellular immunoglobulin (Ig) domains. The virus attachment site in these long molecules is at the N-terminal domain, distal from the plasma membrane. Examples of picornavirus receptors are listed in Table 2.

TABLE 2 Examples of picornavirus receptors

Virus	Receptor	Reference
Major group HRVs	ICAM-1	Greve et al., 1989
Minor group HRVs	LDL-R	Hofer et al., 1994
PVs	PVR (CD155)	Mendelsohn et al., 1989
CVBs	CAR, DAF (CD55)	Milstone et al., 2005 Bergelson et al., 1995
CAV21	ICAM-1	Xiao et al., 2001
CAV9	αVβ3 integrin, αVβ6 integrin	Roivainen et al., 1994 Williams et al., 2004
Echovirus (11 serotypes)	DAF (CD55)	Bergelson et al., 1994, Rossmann et al., 2002
Echovirus 9	αVβ3 integrin	Nelsen-Salz et al., 1999
Echovirus 1	α2β1 integrin	Bergelson et al., 1992
HPEV1	αVβ3 integrin, αVβ1 integrin	Joki-Korpela et al., 2001 Triantafilou et al., 2000
HAV	HAVcr-1	Silberstein et al., 2001
FMDV	αVβ3 integrin, αVβ6 integrin, α5β1 integrin, heparan sulphate	Berinstein et al., 1995 Jackson et al., 2000 Jackson et al., 2000 Jackson et al., 1996
Theiler´s	Sialic acid, VCAM-1	Zhou et al., 1997 Huber et al., 1994

2.3.3 Integrins as picornavirus receptors

2.3.3.1 Integrin structure

Integrins are the principal family of cell surface proteins interacting with the extracellular matrix (ECM). Some of them also act as counter-receptors on adjacent cells (Hynes, 2002). All integrins are noncovalently bound heterodimers, building up from 18 α subunits and 8 β subunits totally 24 different pairs with different ligand specificities and functions. According to the structural data of αVβ3 (Xiong et al., 2001), the heterodimers have an extracellular, globular head (Figure 5). It consists of a seven-bladed β-propeller in the α subunit and an I-like domain (βI) in the β subunit. Of all the α subunits, nine have an additional inserted domain, known as an I (αI) or A domain. Like βI, αI has a common structure of dinucleotide binding or Rossman fold, consisting of a core of β-sheets surrounded by amphipathic α-helices (Lee et al., 1995). A metal ion-dependent adhesion site (MIDAS) at the ligand-binding interface is present in both αI and βI domains. The α subunit leg piece is composed of three sandwich-like domains termed thigh, calf-1 and calf-2. A flexible joint, a genu, locates between thigh and calf domains. The βI domain is inserted in the hybrid domain followed by the PSI (for plexins, semaphorins and integrins), four epidermal growth factor (EGF) domains and a β-tail domain (βTD). The collagen receptor integrins (α1β1, α2β1, α10β1 and α11β1) contain an additional C-helix, protruding from the upper surface of the αI domain (Emsley et al., 2000). Each subunit also has a single transmembrane domain and a short cytoplasmic tail.

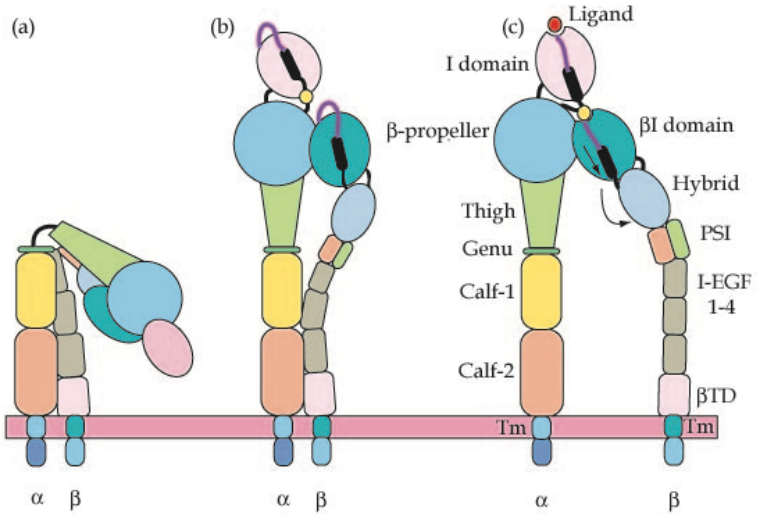


FIGURE 5 Integrin structure and three conformational states with different ligand-binding affinities. a) Low-affinity state, bent with closed headpiece. b) Priming state, extended with closed headpiece. c) High-affinity state, extended with open headpiece. Modified from Luo and Springer, 2006.

2.3.3.2 Integrin activation

A central feature of the integrin receptors is their capacity to direct adhesion temporally and spatially. Integrin adhesiveness is dynamically regulated. Interactions of the integrin cytoplasmic tails with different intracellular proteins lead to conformational changes in the ligand-binding site in the ectodomain (inside-out signaling) and increased ligand-binding affinity. Ligand binding, in turn, induces conformational changes that are transmitted back to the cell resulting in diverse responses (outside-in signaling) (Kim et al., 2003). Attaching to multivalent ECM ligands leads to clustering of integrin receptors, which increases the number of bonds participating in the interaction (valency) (Kim et al., 2004).

Integrins are not active all the time, which is very important for their biological activities, especially platelet function. A number of recent studies has established the existence of at least three conformational states in the integrins: a bent conformer, an extended conformer with a closed headpiece and an extended conformer with an open headpiece (Takagi et al., 2002; Kim et al., 2003; Xiao et al., 2004) (Figure 5). In the bent, low-affinity conformation, with the headpiece folded over the tailpiece, the integrin is not able to bind to biological ligands. Interaction of the membrane-proximal regions of α and β cytoplasmic tails through a salt bridge is believed to keep integrins in the inactive state (Liddington and Ginsberg, 2002). Intracellular talin binding disrupts the salt bridge and leads to tail separation hence activating the integrins (Vinogradova et al., 2002). Concomitant alterations in the transmembrane helices of the integrin subunits take place. Upon activation, the integrin extracellular domains undergo a switchblade-like extension at the genu region (Beglova et al., 2002), representing the conformation with the highest affinity for ligand. In between, the extended but closed conformation is assumed to have an intermediate affinity for ligand (Takagi et al., 2002). The αI domains are known to exist in several configurations with different affinities as well (Emsley et al., 2000; Shimaoka et al., 2003).

2.3.3.3 Biological functions of integrins

2.3.3.3.1 Extracellular matrix adhesions

The ECM is a complex network of matrix molecules, including fibronectin, collagens, laminins and nonmatrix molecules, such as growth factors. Cell adhesion to the ECM induces formation of specialized cell surface structures, termed cell-matrix adhesions. Most of the adhesions are organized by integrins, which link the ECM to the actin cytoskeleton at the cell interior. The ECM provides support but also profound control over cells regulating numerous cellular processes, such as development, wound healing, cell proliferation, cell survival and tumorgenesis. Aberrations in the integrin adhesion functions can lead to pathological conditions, such as thrombosis, inflammation and cancer (Wehrle-Haller and Imhof, 2003).

The ability of cells to modify matrix adhesions in response to alterations in the molecular composition and physical forces present in the ECM, depends on the dynamic coupling with the actin cytoskeleton (Wozniak et al., 2004). The size, morphology and distribution of cell-matrix adhesions can be heterogenous. Assembly of dynamic focal complexes, which form in the protrusions of spreading and migrating cells, is stimulated by small GTPases Rac and Cdc42, (Nobes and Hall, 1995). If stabilized, the focal complexes can mature into larger focal adhesions, which require GTPase Rho activity (Hotchin and Hall, 1995). The focal adhesions can in turn transition into fibronectin and α5β1 integrin containing fibrillar adhesions (Pankov et al., 2000).

The best-characterized cell-matrix adhesions are the focal adhesions. An initial step in their formation involves clustering of integrins following binding to multivalent ECM proteins (Miyamoto et al., 1995). As the cytoplasmic tails of integrins have no intrinsic kinase activity, by clustering they are able to recruit adaptor and signaling molecules to control adhesion-dependent processes. Mature focal adhesions may contain more than a 100 different proteins (Zamir and Geiger, 2001). Talin is a major actin-associating protein, which directly binds to integrin and regulates its activation (Calderwood, 2004). Adaptors and scaffolding proteins, including vinculin and paxillin, link integrin-associated proteins to the actin cytoskeleton. They can also recruit focal adhesion kinase (FAK) to the site (Mitra et al., 2005). FAK is a key signaling component in focal adhesions, which upon activation is autophosphorylated in Y397 creating a high-affinity binding site for Src family kinases (Schaller et al., 1994). Many components of the focal adhesions are multidomain molecules capable of interacting with several partner molecules. Thus, a great number of combinations of molecular interactions are involved in the integrin-actin connection, which constitutes a core machinery for information handling and cell motility.

The focal adhesions are dynamic structures, which assemble, disassemble and translocate in response to cell spreading, migration and division. FAK and Src are important regulators of focal adhesion turnover (Wozniak et al., 2004). Protease calpain activity, which will be discussed more thoroughly later, contributes to the ability of FAK to regulate matrix turnover (Westhoff et al., 2004).

2.3.3.3.2 Integrin trafficking

Integrin endocytosis regulates cell adhesion, spreading and migration. During migration cells form new adhesions at their front and break old adhesions at the rear. Cells perform this by continual turnover of focal adhesions and rearrangement of actin cytoskeleton, but how vesicular transport drives integrins towards the leading front is not completely clear. Previous models suggest that it occurs by continual endocytosis of integrins at the rear of the cell and recycling to the leading front of cell (Bretscher, 1992; Bretscher, 1996). However, the existing evidence supports a different trafficking model (Figure 6). The dynamin-mediated dissassebly of focal adhesions is coupled to endocytosis of clustered integrins (Ezratty et al., 2005). Internalization of β1 integrins is regulated by protein kinase Cα (PKCα) (Ng et al., 1999a), which binds directly to β1 integrin cytoplasmic tails (Parsons et al., 2002). Internalized β1 integrins follow a route typical for many recycling proteins. First they are targeted to early endosomes and subsequently to recycling endosomes, where they colocalize with GTPases ARF6 and Rab11 (Powelka et al., 2004). The prevailing effects of ARF6 are delivery of recycling vesicles to the plasma membrane (D'Souza-Schorey et al., 1998) and regulation of the actin cytoskeleton (Randazzo and Hirsch, 2004). Rab11 associates with recycling endosomes and acts in tethering and fusion of membrane vesicles (Savina et al., 2005). From the recycling endosomes, ARF6 regulates distribution of β1 integrins into membrane lamellipodia (Powelka et al., 2004), which are formed by Rac1 activity (del Pozo et al., 2004). In the lamellipodia, which are the sensing organelles of cells, the initial matrix probing occurs by synchronous movement of activated but unligated β1 integrin clusters with polymerizing actin (Galbraith et al., 2007).

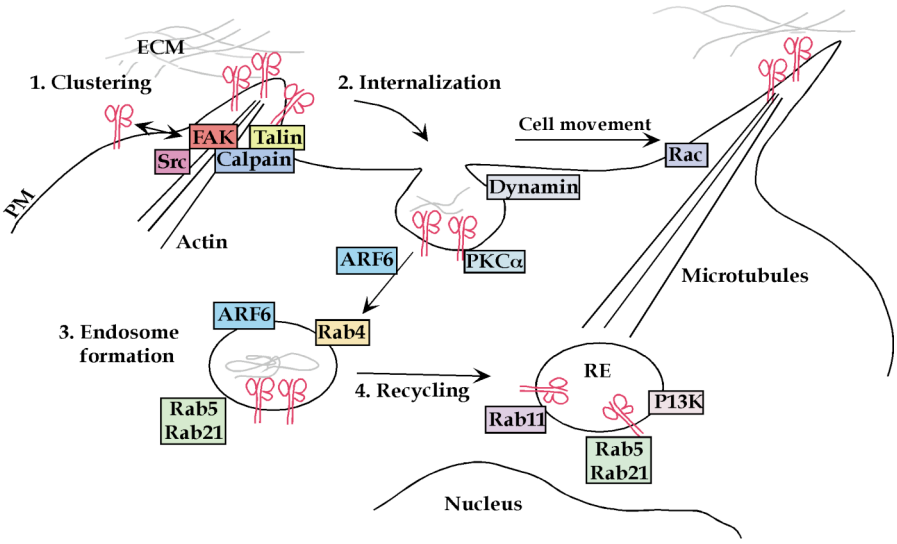


FIGURE 6 Integrin trafficking. Disassembly of ECM adhesions is coupled to integrin endocytosis. Integrin internalization in the endosomal compartments is regulated by dynamin and PKCα. From Rab11 positive recycling endosomes (RE) integrins are recycled back to the base of lamellipodia. The activities of GTPases are shown. Modified from Pellinen and Ivaska, 2006.

Integrin α-chain has a role in integrin internalization also. A recent paper shows that GTPase Rab21 associates with α2 integrin cytoplasmic domains and controls endosomal traffic of β1 integrins (Pellinen et al., 2006). The microtubules mediate the internalization of β1 integrins into large vesicles, which are positive for Rab21 and Rab5 and can contain also caveolin (Pellinen et al., 2006).

2.3.3.3.3 Integrin signaling

Understanding of the structural basis for integrin signaling has increased in the past few years. Ligand binding promotes separation of the transmembrane and cytoplasmic domains of α and β integrin subunits (Kim et al., 2003). Separation of integrin legs creates binding sites for signaling proteins, which bind generally to the integrin β tails. Less is known about signaling mediated by the integrin α tail (Liu et al., 2000). Integrins interact also laterally with other transmembrane proteins, such as members of transmembrane-4 superfamily (TM4SF). TM4SF proteins may link integrin α chains to the intracellular signaling molecules (Zhang et al., 2002). Integrin engagement induces several signaling cascades, which can interact and coordinate each other (Figure 7). Phosphorylation of kinases and elevation of intracellular ions take place upon integrin ligation (Kwon et al., 2000; McCarty et al., 2004). In addition, integrins co-operate with and transactivate growth factor receptors to control cell behaviour (Moro et al., 2002; Yamada and Even-Ram, 2002).

Focal adhesions are the key sites transducing signals to the downstream pathways, including mitogen-activated protein kinases (MAPKs). MAPKs contribute to cell survival and proliferation. There are three major classes of MAPKs: 1) extracellular signal regulated kinase 1/2 (ERK1/2), which associates principally with cell proliferation; 2) c-jun N-terminal kinase (JNK); and 3) p38 kinase, which is activated in response to cellular stress. An alternative link between integrins β1 and αV and MAPKs has been proposed, in which the α subunits mediate interaction of Shc with caveolin (Wary et al., 1996). Active PKC resides in focal adhesions and is thus spatially located to phosphorylate proteins, which regulate the actin cytoskeleton (Jaken et al., 1989).

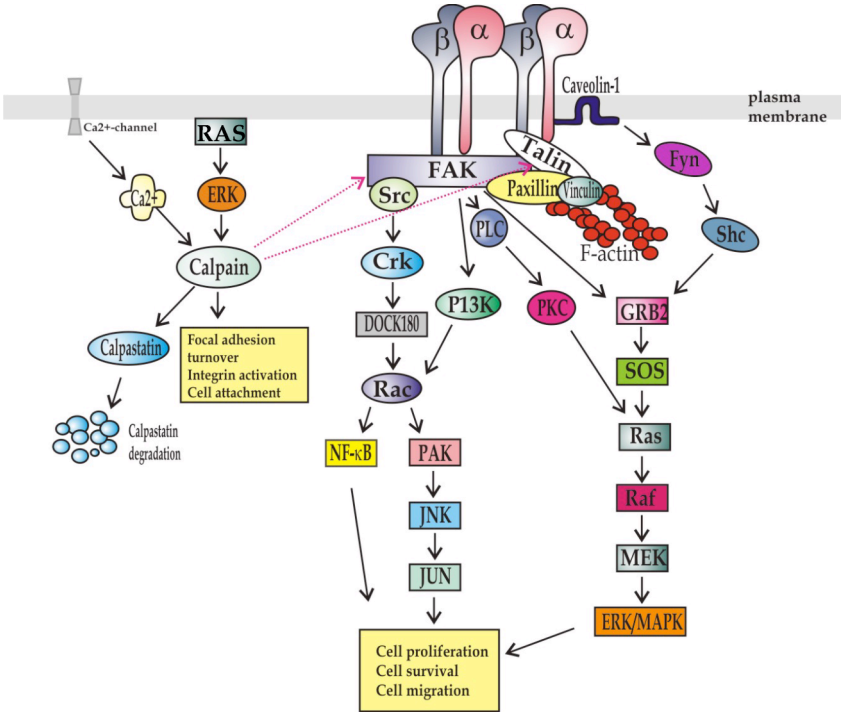


FIGURE 7 Integrin signaling pathways. Ligation of integrins to ECM proteins at focal adhesions leads to clustering of integrins and association with the actin cytoskeleton. Integrins transmit signals from the ECM into various signaling cascades and regulate cellular functions, such as proliferation, migration and survival. Calpains control integrin activation and focal adhesion turnover by modifying target proteins. Modified from Miranti and Brugge, 2002.

2.3.3.4 Integrins as picornavirus receptors

A number of picornaviruses utilize integrins for attachment to the cell surface and for subsequent internalization into the host cells. Viruses may have evolved to recognize cell adhesion receptors because of their relatively low affinity to natural ligands (Wang, 2002). Picornaviruses and natural ligands recognize the same structural units in the integrin receptor but the exact interacting sites are not identical. Integrins are probably favoured by their expression on a wide variety of cells and ability to transmit intracellular signals, which may enhance entry and contribute to the pathogenesis of the infection. In most cases the integrin subunit dimer seems to contain the αV chain. The arginine-glycine-aspartic acid (RGD) tripeptide was the first sequence identified to bind integrins (Ruoslahti and Pierschbacher, 1987). Several integrins (α5β1, α8β1, αIIbβ3, αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8) recognize RGD motifs present in many extracellular adhesive proteins, e.g. fibronectin, vitronectin, collagen, fibrinogen and von Willebrand factor. Integrins used by picornaviruses are listed in Table 2.

2.3.3.5 α2β1 integrin as a receptor for EV1

Integrin α2β1 is expressed in platelets, epithelial cells, and also on many mesenchymal cell types, including endothelial cells, fibroblasts, chondrocytes and osteoblasts. It acts mainly as a collagen receptor in platelets and a collagen/laminin

receptor in other cell types (Elices and Hemler, 1989).

EV1 binds to the α2I domain of α2β1 integrin for cell entry (Bergelson et al., 1992; Bergelson et al., 1993a). The residues 199-201, 212-216 and 289, critical for EV1 binding, are nonidentical but overlapping with the binding sites of collagen on the α2I domain (King et al., 1995). Furthermore, unlike collagen binding to the integrin, the attachment of EV1 is independent of divalent cations (Bergelson et al., 1993a). The structure of the complex of EV1 and α2I domain has been determined by cryo-electron microscopy (Xing et al., 2004). In the model, EV1 binds α2I domain within the canyon on the virus surface with extensive contacts to the outer canyon wall but not with the inner canyon wall nor in direct contact with MIDAS (Xing et al., 2004). Moreover, the collagen peptide superimposed on the α2I domain crystal structure shows that collagen and virus cannot bind simultaneously, since their binding sites occupy overlapping space. To compete with collagen for free α2β1 integrin receptors, EV1 has affinity ten times greater than the natural ligand for binding (Xing et al., 2004).

The interaction of EV1 with the α2I domain does not induce uncoating of the virus (Marjomäki et al., 2002). However, it is believed to stabilize the virus particle and protect it from premature genome release before the right time and place. In the reconstruction of the virus-receptor complex, the five binding sites on the viral pentamer are able to accommodate the entire integrin heterodimer simultaneously without steric hindrance (Xing et al., 2004). Thus, avidity increase resulting from multiple receptor binding sites on the virus capsid assures very tight, practically irreversible binding. The multiple binding of integrins in sufficient vicinity to each other initiates clustering of the receptors. Clustering, in turn, leads to intracellular signaling which the virus takes advantage of during entry.

2.4 Endocytic routes of picornaviruses

Increasingly sophisticated cell imaging techniques, high-resolution electron microscopy and systems biology (Pelkmans et al., 2005) have uncovered the great diversity of internalization routes (Figure 8). Inert virus particles are dependent on the host physiology and exploit cellular endocytic routes to gain access to the host cell. Trafficking within an endocytic vesicle is advantageous to a virus. It can move easily through intracellular obstacles such as the cortical actin cytoskeleton and crowded cytoplasm with the help of molecular motors. Viruses dependent on low pH for uncoating can escape through endosomal membranes and avoid the hydrolytic lysosomes. In addition, since no viral component is left on the plasma membrane, the immune system does not detect the intruder (Smith and Helenius, 2004).

Viruses can choose between several endocytic pathways, which obviously have multiple connections and intersections with each other (Kirkham et al., 2005; Kirkham and Parton, 2005). Besides the classical clathrin-mediated route, viruses can internalize through non-clathrin routes. These include caveolar-dependent and -independent pathways with perhaps multiple variants and macropinocytosis.

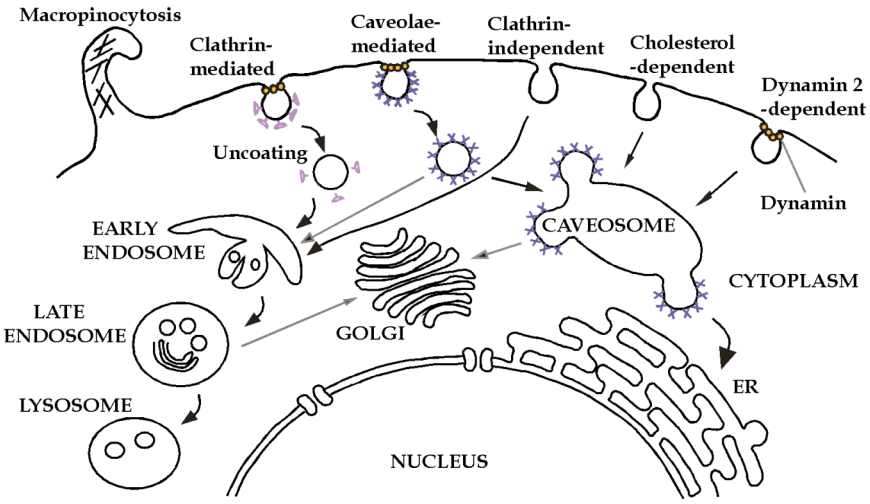


FIGURE 8 Endocytic pathways used by viruses. The pathways may differ in their dependence on clathrin, caveolin and dynamin but can share the intracellular machinery. Modified from Marsh and Helenius, 2006.

2.4.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is responsible for the uptake of nutrients and most plasma membrane receptors undergoing endocytosis (Figure 8.) The cytoplasmic domains of some cargo molecules, such as transferrin receptor (Collawn et al., 1990), contain endocytic signals. They are recognized by adapter protein complex AP-2, which has a role in the assembly of clathrin-coated pits (CCPs) and also in the sorting of cargo (Benmerah and Lamaze, 2007). Following the continual incorporation of clathrin and AP-2, the CCPs form constitutively and take in cargo. They gradually invaginate into free clathrin-coated vesicles through dynamin activity and actin polymerization. In recent years a number of adapter proteins functioning in cargo recognition and clathrin coat assembly have been identified (Traub, 2003; Maldonado-Baez and Wendland, 2006). Also, the existence of different subpopulations of CCPs have been revealed by real-time imaging (Bellve et al., 2006).

In the tubulo-vesicular early endosome (pH 6.2), where the endocytosed receptors and bound ligands are delivered first, efficient sorting occurs. The limiting membrane of early endosomes is built from structurally and functionally different modular elements showing defined biophysical properties (Gruenberg, 2001). Some receptors are recycled back to the plasma membrane for re-use through recycling endosomes, whereas downregulated receptors are trafficked to late endosomes and finally to lysosomes for degradation. Transport from early to late endosomes is mediated by multivesicular intermediates, which fuse selectively with late endosomes (Gu and Gruenberg, 1999). Late endosomes contain cisternal and vesicular regions and often have the appearance of multivesicular bodies. They differ from early endosomes by their lower luminal pH (5.5-5.0) and distinct protein composition (Piper and Luzio, 2001). Most cargo from late endosomes is transported to lysosomes, which contain degradative acid hydrolases. Different endosomal compartments associate with certain GTPases of the Rab family to coordinate the vesicular traffic typical to the organelle.

2.4.2 Caveolae-mediated endocytosis

Various clathrin-independent entry pathways have been identified. They differ, e.g., by their dependence on dynamin. The caveolae-mediated entry pathway is possibly the best-characterized dynamin-dependent entry route (Pelkmans et al., 2001) (Figure 8). Caveolae are flask-shaped pits (50-80 nm) of the plasma membrane. They are marked by the structural proteins caveolin-1 and caveolin-2 in nonmuscle cells, and caveolin-3 in the muscle cells (Way and Parton, 1995). The assembly of the caveolar domains occurs in the Golgi complex, from which they are transported in vesicles to the cell surface. A tight association with the cortical actin cytoskeleton inhibits the lateral movement of caveolae and keeps them as distinct regions (Tagawa et al., 2005). Stimulation by e.g. simian virus 40 (SV40), triggers a transient breakdown of actin filaments, clearing the way for the caveolar vesicles to internalize (Pelkmans et al., 2002). Caveolar pits are formed by caveolins in the specific, ordered lipid domains of the plasma membrane, also called rafts. Whether rafts are pre-existing structures in biological membranes that are self-organized by aggregation of sphingolipids and cholesterol (Simons and Ikonen, 1997), or are induced by clustering of membrane protein components (Gaus et al., 2006), has been controversial. Rafts are postulated to be signaling platforms that attach specific proteins, e.g. GPI-APs, while excluding others (Simons and Ikonen, 1997). However, the size and function of lipid rafts are debated because they are too small to be optically resolved and have no definable ultrastructure. Here, noncaveolar lipid rafts are referred to as GPI-AP enriched domains.

Caveolins are integral membrane proteins, with both the N- and C-termini facing the cytosol. The abilities of caveolin-1 to bind cholesterol and fatty acids directly (Murata et al., 1995; Trigatti et al., 1999), as well as to form oligomers of high molecular weight (Monier et al., 1995) are needed in the formation of caveolae. In addition to the cell surface, caveolin-1 localizes to the cell surface, the Golgi complex, and also partially to recycling endosomes (Gagescu et al., 2000). Caveolar endocytosis is a slow, cargo-triggered event with low capacity. RNA interference of the human kinome has revealed that a remarkable number of kinases participate in endocytosis. Clathrin- and caveolae-dependent endocytosis is affected by 36 kinases. Of these kinases 23 exert opposite effects on the two entry mechanisms, enhancing one pathway at the expense of the other (Pelkmans et al., 2005). Internalized caveolae accumulate in caveosomes, caveolin-1 positive, neutral organelles. They are devoid of markers for early, recycling or late endosomes or the Golgi complex (Pelkmans et al., 2001). Little is known about caveosomes and the involvement of intracellular compartments in the caveolae-mediated pathway. Caveolae can fuse with early endosomes (Parton and Simons, 2007) and, after intersecting with the clathrin-dependent pathway, also be degraded (Botos et al., 2007). Also, whether there are multiple types of caveosomes, which are regulated differently, is unclear.

2.4.3 Other clathrin-independent endocytic routes

GPI-AP enriched domains of the plasma membrane are usually associated with other clathrin-independent endocytic processes, which may differ in their

requirements for dynamin, small GTPases and other regulators (Figure 8). Different entry routes may share common mechanisms, such as cholesterol sensitivity and internalized vesicular intermediates.

Endocytosis of interleukin-2 receptors occurs by a dynamin-dependent, cholesterol sensitive mechanism that is regulated by GTPase RhoA (Lamaze et al., 2001). Internalization of GPI-APs is independent on RhoA and dynamin, but requires the activity of Cdc42 (Sabharanjak et al., 2002). Possibly additional routes exist for the uptake of GPI-APs into cells. For example, endocytic uptake of cholera toxin B (CtxB) can occur by different mechanisms, either by caveolae-mediated or caveolae- and clathrin –independent processes without dynamin (Torgersen et al., 2001).

In cells devoid of caveolae, the internalization of SV40 in small, tight fitting vesicles is quicker and independent on dynamin 2. The vesicles are delivered to structures morphologically and biochemically resembling caveosomes but negative for caveolin. The caveolar -independent route can exist in cells in the presence of caveolin-1 and the parallel routes can merge in caveosomes (Damm et al., 2005).

2.4.4 Macropinocytosis

Macropinocytosis allows cells to sample large amounts of extracellular fluid, antigens and pathogens (Figure 8). Protrusions at the sites of actin-driven membrane ruffling fuse back with the plasma membrane to generate large, heterogenous vesicles, macropinosomes, which are readily labeled with fluid-phase markers (Swanson and Watts, 1995).

Generation of macropinosomes is highly dependent on remodeling of the subcortical actin filaments by GTPases Rac1 and Cdc42 (West et al., 2000; Anton et al., 2003). Remodeling of actin and the proper membranous localization of activated Rac1 requires cholesterol and the activity of PKC (Grimmer et al., 2002). Othe regulators of macropinocytosis encompass p21-activated kinases (PAKs) regulating the uptake process downstream of Rac1 and Cdc42 (Dharmawardhane et al., 2000); PI3K for the closure of macropinosomes (Araki et al., 1996; Araki et al., 2007); and amiloride-sensitive Na+/H+ exchanger (Meier et al., 2002).

In some cell types, such as macrophages and dendritic cells, macropinocytosis is a constitutive process. The forming macropinosomes behave like early endosomes, mature progressively into late endosomes and finally fuse with lysosomes (Racoosin and Swanson, 1993). In other cell types the process is pronounced after growth factor stimulation, which induces formation of recycling macropinosomes (West et al., 1989; Nobes et al., 1995; Bryant et al., 2007). Maturation of macropinosomes occurs by the recruitment of sorting nexins (SNXs) (Kerr et al., 2006). The SNXs are a group of hydrophilic proteins, which regulate cargo trafficking in the endosomal system (Teasdale et al., 2001). They are associated with discrete microdomains of the macropinosome and involved in the formation and extension of tubular structures. When the tubules separate from the macropinosome, unwanted components, such as pathogens, are removed from the organelle. Following the departure of the tubules, the remaining macropinosomes continue maturing and eventually, fuse with lysosomes (Kerr et al., 2006).

2.4.5 Entry routes used by picornaviruses

For many picornaviruses, one or more of a diverse set of endocytic routes have been described. New entry mechanisms are idenfied continuously and hopefully they increase our understanding of viral entry. Studying of viral entry may be complicated, since viruses may use parallel or alternative pathways depending on the cell type, virus multiplicity and growth conditions. Some examples of picornavirus entry routes are described in Figure 9.

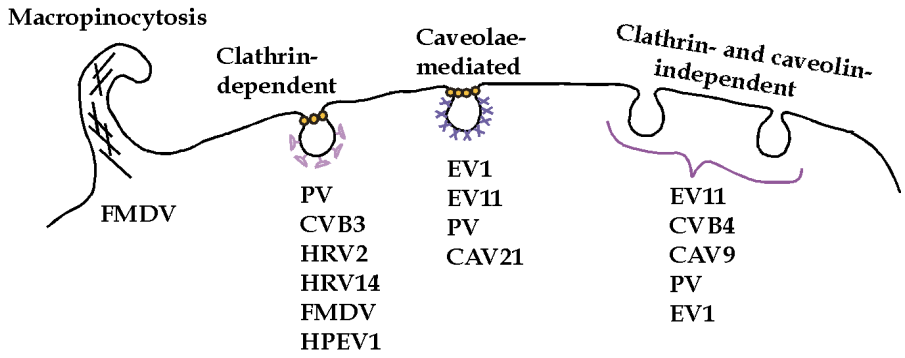


FIGURE 9 Examples of picornavirus entry routes. The clathrin-dependent pathway is used by PV (Kronenberger et al., 1998), CVB3 (Chung et al., 2005), HRV2 (Snyers et al., 2003), HRV14 (Grunert et al., 1997; Kronenberger et al., 1998), FMDV (O'Donnell et al., 2005) and HPEV1 (Joki-Korpela et al., 2001). The caveolae-mediated pathway is used by EV1 (Marjomäki et al., 2002), EV11 (Stuart et al., 2002), PV (Coyne et al., 2007) and CAV21 (Shafren, 1998). Variants of the clathrin- and caveolin – independent pathways are used by EV11 (Stuart et al., 2002), CVB4 (Triantafilou and Triantafilou, 2004), CAV9 (Triantafilou and Triantafilou, 2003), PV (Brandenburg et al., 2007) and EV1. Modified from Marsh and Helenius, 2006.

2.5 Calpains

Calpains are a family of cytosolic cysteine proteases present in all animal cells. Their enzymatic activity is dependent on the high intracellular Ca^{2+}. The human calpain family has 14 members which are widely expressed both in tissue-specific and ubiquitous manner (Goll et al., 2003; Suzuki et al., 2004). The two ubiquitously expressed isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calpain), are the best-characterized members as well as the endogenous inhibitor of their proteolytic activity, calpastatin. Calpain 2 is the major form expressed in several tissues whereas calpain 1 is expressed in neuronal cells, erythrocytes, T lymphocytes and platelets (Sato and Kawashima, 2001). A number of calpain homologues have been found in other organisms such as fungi and yeast.

Despite over 40 years of research, the physiological function of the calpains in normal cells has remained largely a puzzle. Calpains 1 and 2 are vital for many cellular processes, including adherence, migration, some pathways of apoptosis, cell cycle, regulation of gene expression and different signal transduction pathways. Calpains 1 and 2 are critically important for normal development, since transgenic mice devoid of the regulatory subunit are embryonically lethal (Arthur

et al., 2000). At the pathological level, the calpains have been implicated in diabetes, cataracts, cancer, multiple sclerosis, muscular dystrophies and Alzheimer´s disease (Zatz and Starling, 2005). Viral infections also tend to activate calpains.

2.5.1 Calpain structure

The crystallographic structure of human calpain 2 has been solved in a Ca^{2+} -free state (Strobl et al., 2000), which has shed remarkable light on the understanding of activation and regulation of calpains. Calpains 1 and 2 are heterodimers composed of a unique, large 80 kDa catalytic subunit and a common small regulatory subunit (Goll et al., 2003) (Figure 9). The catalytic subunit has four domains (dI-dIV), whereas the regulatory subunit has two domains (dV-dVI) (Strobl et al., 2000; Sorimachi and Suzuki, 2001). The regulatory subunit assists the calpain molecules to fold correctly and maintains a complete calpain/calpastatin network (Arthur et al., 2000). Domain dI is an N-terminal single α-helix that can interact with dVI, thereby stabilizing the protein in a circular arrangement. It also serves as an anchoring helix through interaction with dV. Domain dII contains the catalytic triad Cys105, His262 and Asn286 and can be further divided into subdomains dIIa and dIIb. Domain dIII consists of eight β strands and is structurally related to C2 domains, Ca^{2+}-regulated phospholipid binding sites of various kinases, including PKC (Tompa et al., 2001). An acidic loop of ten negatively charged amino acids within dIII may have a role in the Ca^{2+}-dependent activation of calpain (Strobl et al., 2000). Like dIV, dVI is a Ca^{2+}-binding domain. They both contain five EF-hand motifs with the fifth EF-hand from each subunit interacting with the other to form heterodimers (Lin et al., 1997; Hosfield et al., 1999). Domain dV, containing hydrophobic residues as well as glycine residues, is largely unresolved in the crystal structure.

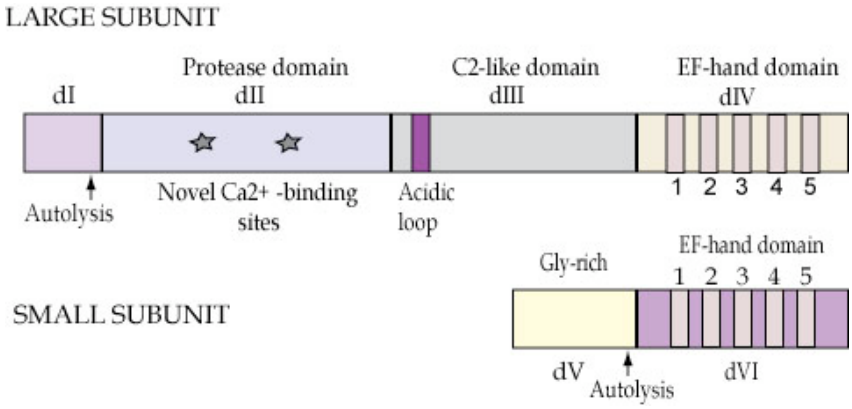


FIGURE 10 Calpain structure. The large subunit is divided into four domains and the small subunit, into two domains. Protese activity is contained in domain II, domain III contributes to phospholipid binding and phosphorylation, EF-hand domains are the sites of Ca^{2+} binding. Novel Ca^{2+}-binding sites are indicated by stars and sites of autolysis by arrows. Modified from Franco and Huttenlocher, 2005; Khorchid and Ikura, 2002.

2.5.2 Calpain activation

The activation and regulation of calpain *in vivo* is a complex process of which details are only now beginning to be resolved. The best-characterized mechanism is activation by Ca^{2+} and because of this, the name calpain was derived (Guroff, 1964). However, calpain 1 and 2 differ greatly from their *in vitro* Ca^{2+} requirements for activation: calpain 1 needs 5-50 μM concentrations, whereas calpain 2 is activated by 200-1000 μM concentrations of Ca^{2+}. In general, these concentrations of Ca^{2+} appear to be unattainable intracellularly (50-300 nM). Additional factors, such as binding to phospholipids (Saido et al., 1992; Arthur and Crawford, 1996; Shao et al., 2006) or activation factors (Melloni et al., 2000), interaction with calpastatin (Tompa et al., 2002; Averna et al., 2006), autoproteolysis of the subunits (Baki et al., 1996; Michetti et al., 1996; Elce et al., 1997; Guttmann et al., 1997; Suzuki and Sorimachi, 1998) or phosphorylation (Goll et al., 2003; Glading et al., 2004; Xu and Deng, 2006) have been suggested to participate in lowering of the Ca^{2+} requirement.

Calpains and calpastatin are colocalized in cells (Kumamoto et al., 1992; Goll et al., 2003). Once activated by the rise in Ca^{2+} concentration, calpains have been described to translocate from the cytosol to the plasma membrane or from focal adhesions to the vicinity of their target proteins (Beckerle et al., 1987; Fox et al., 1993; Sato and Kawashima, 2001; Averna et al., 2006). Because of the gel-like composition of the cytoplasm, random diffusion of calpains seems not sufficient for locating substrates in different subcellular sites (Hood et al., 2006). Likely, calpains are not solely free cytoplasmic proteins, but associated with membranes in significant amounts. In accordance, calpains and calpastatin have been shown to associate with the ER, Golgi complex, plasma membrane and GPI-AP enriched domains (Morford et al., 2002; Hood et al., 2003; Kifor et al., 2003; Hood et al., 2004; Nuzzi et al., 2007), with calpain 2 as the prevailing isoform in the organelles (Hood et al., 2003). Association of calpains and calpastatin with the organelles likely requires hydrophobic interactions via phosphoinositide biphosphate (PIP_2) (Strobl et al., 2000) and the regulatory subunit of calpains (Hood et al., 2006).

The puzzling feature of calpain activation can be partly explained by localization of calpains and calpastatin to the ER and Golgi complex. In both of these compartments Ca^{2+} concentration increases remarkably following proper cell signaling (Pinton et al., 1998; Ashby and Tepikin, 2001). An appropriate signaling event, such as integrin ligation, leads to a rise in Ca^{2+} concentration, which in turn may induce conformational change (Moldoveanu et al., 2004) and activation of calpain. In association with the organelles, calpain 2 is able to modify newly synthesized proteins in the ER or existing actin-associated proteins proximal to the plasma membrane. Translocation of calpain 2 to the GPI-AP enriched domains occurs by incorporation of the molecule into the Golgi-derived vesicles, which fuse with the plasma membrane (Hood et al., 2003; Hood et al., 2006). Clustering of β1 integrin containing GPI-AP enriched domains into functional focal contacts allows calpain 2 to participate in the reorganization of the actin cytoskeleton. Calpain 2 avoids inhibition of calpastatin by residing in its nonautolyzed form within the ER and the Golgi lumen, whereas active calpains 1 and 2 are found in the cytosolic sides of the organelles in the calpastatin-rich environment (Hood et al., 2004).

Calpastatin, which needs a lower Ca^{2+} concentration to inhibit calpain 2 activity than calpain 2 requires for activation (Goll et al., 2003), is not able to bind calpain 2 prematurely. Calpains lack any known N-terminal signal sequence to target to the ER or Golgi complex. However, calpains are suggested to possess an internal, post-translationally formed topogenic signal, which may guide calpains into the lumen of the organelles (Hood et al., 2006).

2.5.3 Calpain function

More than one hundred proteins have been identified as calpain substrates *in vitro*. The target molecules of calpain 1 and calpain 2 seem to be the same, most of them being actin-associated or cytoskeletal proteins (Bialkowska et al., 2000; Yan et al., 2001), signaling molecules, such as FAK and PKC (Kishimoto et al., 1989; Carragher et al., 1999), and GTPases (Kulkarni et al., 2002). As a result of calpain processing, the activity of PKC is downregulated (Kishimoto et al., 1989), which applies also to the activities of FAK and Src. Some enzymatic activities, such as protein tyrosine phosphatase 1B activity (PTP-1B), are up-regulated by calpain cleavage (Sato and Kawashima, 2001). Calpain 1 and calpain 2 may respond to various cellular signals differently, otherwise they are thought to perform similar physiological functions.

Calpains were first connected to integrin-ligand interactions by studies with platelets, in which activated calpain 1 was shown to cleave cytoskeletal talin, filamin and spectrin (Fox et al., 1983; Fox et al., 1993; Schoenwaelder et al., 1997). Afterwards, calpains were observed to degrade the cytoplasmic domains of β integrins, thus regulating integrin-cytoskeletal interactions (Pfaff et al., 1999). By modifying integrin-related proteins calpains contribute to different processes, such as clustering of integrins (Bialkowska et al., 2000; Yan et al., 2001) and spreading of cells (Kulkarni et al., 2002). In the integrin-mediated migration, calpains have been implicated in both adhesion formation (Kulkarni et al., 1999; Franco et al., 2004) and rear detachment (Huttenlocher et al., 1997).

Calpains have no clearly definable cleavage site specifity, which explains the abundance of substrates and cellular roles. Calpains merely modify, rather than digest the target molecules producing fragments with different activities from those of their intact forms. Recognition and proteolysis seem to be governed by preferences to certain amino acids, secondary structure and PEST (Pro, Glu/Asp, Ser/Thr) region, but to a large extent, calpains recognize other high order structured elements also (Tompa et al., 2004).

2.5.4 Calpains and viruses

Many viral infections are known to activate calpain protease activity. In picornaviruses, efficient inhibition of PV $2A^{pro}$ protease activity by calpain inhibitor 1 has been observed (Molla et al., 1993). Calpain 1 is also known to participate in the inhibition of HIV replication in latently infected cells. The target of calpain 1 is an inhibitor of HIV gene regulation, NF-κB (necrosis factor-κB) (Teranishi et al., 2003). The molecule contains PEST sequence, which calpain 1 possibly recognizes. In HCV infection the nonstructural protein NS5A, which first

activates calpains 1 and 2, is itself processed by them (Kalamvoki and Mavromara, 2004). Both calpain 1 and calpain 2 activity is involved in human cytomegalovirus (HCMV) infection, in which the target of the proteases is an inhibitor of cyclin-dependent kinases (Chen et al., 2001). The substrate protein is rich in PEST amino acids also. Finally, calpain 1 participates in apoptosis in reovirus-infected cells (Debiasi et al., 1999).

The detailed mechanisms of the calpain action remain unsolved. Calpains are complex proteins that may act co-operatively. Addressing the pathophysiological functions of calpains, especially *in vivo*, has been difficult. Studies have been done using mice lacking or overexpressing calpastatin, the only calpain-specific inhibitor. The alteration of the calpastatin expression causes no defect in the mice under normal conditions. Calpastatin thus seems to function as a negative regulator of calpains only under pathological states (Takano et al., 2005). Also other proteolytic systems may compensate for decreased calpain activity upon calpain inhibition (Razeghi et al., 2007).

Since the calpains are mediating the effects of several viral infections, they might serve as excellent targets of therapeutic interventions. By acting on the activated form of calpain, the antivirals would minimize damage to the host organism and relieve patients´ symptoms.

3 AIM OF THE STUDY

EV1 is a representative of the picornavirus family, whose members are related structurally and genetically. Despite this similarity, picornaviruses have evolved to use a diverse collection of cell surface molecules as their receptors, and thus, often internalize by different means. To characterize the life cycle of EV1 in detail the specific aims of this research were:

1. To study the interaction of EV1 with its receptor and the entry mechanism of the virus-receptor complex.

2. To study the signaling events associated with entry.

3. To study the host factors needed for replication of EV1.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Cells, viruses and antibodies (I-IV)

SAOS cells (ATCC), which do not normally express the α2 integrin subunit, were stably transfected with an expression construct encoding α2 integrin (SAOS-α2β1 cells) (Ivaska et al., 1999). The α2/α1 integrin mutant was a chimeric α2 subunit with α1 integrin cytoplasmic domain (SAOS-α2/α1β1 cells) (Ivaska et al., 1999). SAOS-pAW cells, which contain an empty expression vector, were used as a control. Other cells lines used were CV-1, GMK, A549 and 293 cells, all from ATCC. HeLa MZ cells were from Dr. Urs Greber, University of Zurich, Switzerland. The cells were maintained in Dulbecco`s MEM (DMEM, Gibco), supplemented with 10% foetal calf serum or foetal bovine serum (FCS/FBS), 2 mM L-glutamate and 1% penicillin/streptomycin. For transfected SAOS cells G418 (50 mg/ml) was added to maintain the integrin expression. For infections, the concentration of serum in the medium was reduced to 1%.

EV1 (Farouk strain, ATCC) was propagated in GMK cells and purified in sucrose gradients as described previously (Abraham and Colonno, 1984), whereas HPEV1 (Hyypiä et al., 1992) was grown in A549 cells and CVB3 (Vuorinen et al., 1994) in LLC-MK_2 cells. Purified SV40 was obtained from L. Pelkmans (Pelkmans et al., 2001).

Fluorescent labeling of purified EV with Texas Red-X or Alexa Fluor 594 (Molecular Probes) and SV40 with Cy5, FITC or Alexa Fluor 594 dyes was performed as described earlier (Pelkmans et al., 2001). Table 3 describes the primary antibodies used in this thesis.

TABLE 3 Primary antibodies used in this thesis

Antibody	Target	Source	Provider/Reference
EV1 antiserum	EV1 capsid proteins	rabbit	Marjomäki et al., 2002 (I)
HPEV1 antiserum	HPEV1 capsid proteins	rabbit	Joki-Korpela et al., 2001
CVB antiserum	CVB3 capsid proteins	rabbit	Vuorinen et al., 1994
12F1	integrin α2 subunit	mouse	BD Pharmingen
MAB1950	integrin α2 subunit	mouse	Chemicon
MCA2025	integrin α2 subunit	mouse	Serotec
HAS6	integrin α2 subunit	mouse	Dr. Fiona M. Watt
L230	integrin αV subunit	mouse	Dr. Jyrki Heino
Caveolin-1	caveolin-1	mouse	Zymed
Caveolin-1 (2234)	caveolin-1	mouse	Transduction Laboratories
Caveolin-1	caveolin-1	rabbit	Transduction Laboratories
Caveolin-1 (N-20)	caveolin-1	rabbit	Santa Cruz
1D3	protein disulfide isomerase (PDI)	mouse	Huovila et al., 1992
Syntaxin 17	smooth ER	goat	Steegmaier et al., 1998
AB730	β2 microglobulin	rabbit	Chemicon
BM-63	β2 microglobulin	mouse	Sigma
W6/32	class I HLA	mouse	Barnstable et al., 1978
HA	hemagglutinin	mouse	Santa Cruz
Myc	myc peptide	mouse	Suomalainen and Garoff, 1994
PKC	protein kinase C	mouse	Upstate Biotechnology
pPKC	phosphorylated protein kinase C	rabbit	Upstate Biotechnology
ERK	extracellular signal regulated kinase	rabbit	Santa Cruz
Tubulin	tubulin	mouse	Sigma
EEA-1	early endosomal antigen-1	rabbit	Mu et al., 1995
CI-MPR	cation-independent mannose-6-phosphate receptor	rabbit	Marjomäki et al., 1990
p230	*trans*-Golgi network	mouse	Transduction Laboratories
GB2	*trans*-Golgi nework protein 46 (TGN-46)	rabbit	Banting et al., 1998
p23	*cis*-Golgi network	rabbit	Rojo et al., 1997
CD63	late endosomes/ lysosomes	mouse	Zymed
3D antiserum	CVB3 3D RNA polymerase	synthetic peptide	Auvinen et al., 1993, Karjalainen and Kakkonen, unpublished
Calpain 1	calpain 1	mouse	Calbiochem
Calpain 1	calpain 1	rabbit	Santa Cruz
Calpain 2	calpain 2	rabbit	Sigma
Transferrin	transferrin	rabbit	Zymed and Behring Institute

4.2 Immunofluorescence and confocal microscopy (I-IV)

Subconfluent cell cultures were incubated with viruses for 1 h in cold, washed and incubated at 37°C for various time periods. The cells were fixed with cold methanol at -20°C for 6 min or with 4% paraformaldehyde for 20 min at room temperature. The cells were quenched with 50 mM NH_4Cl and permeabilized with 0.2% Triton X-100, 0.05% saponin or 0.3% TX-100 before immunofluorescence labeling. In addition to antibodies, lysotracker Red DND99 (100 nM, Molecular Probes) was used to mark the lysosomes and holo-transferrin (I mg/ml, Sigma) to detect the clathrin route. Table 4 describes the inhibitors used in this thesis. Highly cross-absorbed goat secondary antibodies against rabbit (red, 546 nm; Molecular Probes) and mouse (Alexa green, 488 nm; Molecular Probes) immunoglobulins were used. For the triple immunofluorescence labeling, Zenon rabbit IgG labeling kit (Molecular Probes) was used.

TABLE 4 Inhibitors used in this thesis

Inhibitor	Provider	Concentration	Effect
Brefeldin A	Sigma	0.5-2 μg/ml	Disruption of Golgi
Bisindolylmaleimide	Sigma	5 μM	Inhibition of PKC
Cytochalasin D	Sigma	1-7 μg/ml	Inhibition of actin polymerization
Latrunculin A	Molecular Probes	0.13-1 μM	Sequestering of actin monomers
Jasplakinolide	Molecular Probes	0.1-0.75 μM	Stabilization of actin polymers
Methyl β-cyclodextrin	Sigma	10 mM	Extraction of cholesterol
Nocodazole	Sigma	33 μM	Disruption of microtubules
Nystatin	Sigma	25 μg/ml	Sequestering of cholesterol
Progesterone	Sigma	10 μg/ml	Inhibition of cholesterol synthesis
Okadaic acid	Sigma	1 μM	Inhibition of serine and threonine phosphatases
Sodium orthovanadate	Calbiochem	1 mM	Inhibition of tyrosine phosphatases
Calpeptin	Calbiochem	140 μM	Inhibition of calpain
Calpain inhibitor 1	Roche	130 μM	Inhibition of calpain 1
Calpain inhibitor 2	Roche	130 μM	Inhibition of calpain 2
Antipain	Sigma	125-1000 μM	Inhibition of trypsin-like and cysteine proteases, papain
Leupeptin	Sigma	125-1000 μM	Inhibition of trypsin-like and cysteine proteases
Aprotinin	Sigma	1.25-10 U/ml	Inhibition of e.g. serine proteases and chymotrypsin
Trypsin inhibitor	Sigma	0.5-4 mM	Inhibition of trypsin
Elastatinal	Sigma	62.5-500 μM	Inhibition of elastase-like serine proteases

The cells were examined with an Axiovert 100 M SP epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a confocal setup (Zeiss LSM510). Images were acquired using a Plan Neofluar objective (63x NA 1.25, oil) and a digital resolution of 512 x 512. False colocalization signals were avoided by scanning fluorescence from different excitation wavelengths separately.

4.3 Transfections and siRNA mediated protein depletion (I-IV)

The transient transfections with Fugene (Roche or Boehringer Mannheim) or Superfect (Qiagen) were performed according to the manufacturers´ instructions. Table 5 describes the constructs (WT; wild type, DN; dominant negative, CA; constitutively active) used in this thesis.

TABLE 5 Constructs used in this thesis

Construct	Provider/Reference	Target
HA-tagged caveolin-3 (WT)	Roy et al., 1999	Caveolin-3
HA-tagged caveolin-3^{DGV} (DN)	Roy et al., 1999	Caveolin-3
GFP-caveolin-1 (DN)	Pelkmans et al., 2001	Caveolin-1
Caveolin-1-GFP (WT)	Pelkmans et al., 2001	Caveolin-1
GFP-tagged dynamin 2 (WT)	Cao et al., 1998; Ochoa et al., 2000	Dynamin 2
GFP-tagged dynamin 2 (DN; K44A)	Cao et al., 1998; Ochoa et al., 2000	Dynamin 2
Myc-tagged AP180C (DN)	Ford et al., 2001	Clathrin
GFP-tagged Eps15 (WT; DIIId2)	Benmerah et al., 1999	AP-2 complex
GFP-tagged Eps15 (DN; d95/925)	Benmerah et al., 1999	AP-2 complex
GFP-tagged actin	CLONTECH	Actin
PKCα (WT)	Ng et al., 1999b	PKCα
PKCα (DN; kinase-dead, substrate-binding form)	Mostafavi-Pour et al., 2003	PKCα
PKCε (DN; kinase-dead form)	Ivaska et al., 2002	PKCε
MEK (DN; 1E8)*	Holmström et al., 1999	MEK
MEK (CA; 1R4F)*	Holmström et al., 1999	MEK
Ras (DN; asn-17-Ras)*	Feig and Cooper, 1988	Ras

*) co-transfected to the cells with pEGFP-C2 (CLONTECH)

siRNA duplexes were synthesized by Qiagen. The target sequences for human calpain 1 (GenBank accession number NM 005186) siRNA were CCG GAC CAT CCG CAA ATG GAA (calpain 1-1) and TAG GAT CAT CAG CAA ACA CAA (calpain 1-2). The target sequences for human calpain 2 (GenBank accession number NM 001748) siRNA were CCC GAG AAT ACT GGA ACA ATA (calpain 2-1) and TCG GCT GGA AAC GCT ATT CAA (calpain 2-2). A non-targeting scramble siRNA (Eurogentec) was used as a control. siRNAs were cotransfected into cells with siGLO transfection indicator (Dharmacon) using Oligofectamine (Invitrogen) based on the manufacturer´s instructions with modification. Briefly, 4 μl of oligofectamine and 15 μl of serum-free DMEM (Gibco) were incubated for 10 min. The mixture was combined with a

mixture containing total 20 μl of siRNA (20 μM) and 175 μl of serum-free DMEM and incubated for 20 min before addition to cells on glass coverslips. After 4 h incubation at 37°C, the cells were supplemented with 30% DMEM. Transfected cells were incubated for 72 h before use in infections. The amount of infected cells was calculated among transfected cells based on the siGLO staining in nucleus. Additionally, the RNA content was assessed by TaqMan reverse transcription RT-PCR and was related to the amount of RNA produced by β-actin.

4.4 Live cell and real-time fluorescence microscopy (II, III)

For live cell microscopy cells were stained with Alexa 594 –conjugated mutant aerolysin (ASSP) (Fivaz et al., 2002) together with anti-α2 mAb on ice for 1 h. Alternatively, cells transfected with cDNA encoding caveolin-1-GFP or actin-GFP were treated with anti-α2 mAb. After washes the cells were transferred to a preheated sample stage (27/37°C) of the confocal microscope (Zeiss Axiovert 100M with LSM510). Anti-mouse Alexa 488 (aerolysin-containing cells) or anti-mouse Alexa 546 (GFP-containing cells) was added and Z-stacks through the cells were imaged at 5- or 10-min intervals. CO_2-independent medium (Sigma) was used. 3D projections of the selected images were created for each time point using LSM510 3.0 and AxioVision Inside4D 3.0 (Zeiss). These image series were processed and edited using Corel Photo-Paint 8 and Quick Time Pro.0.2.

For real-time fluorescence microscopy AF-EV1 was bound to untransfected or GFP-caveolin-1 transfected CV-1 cells for 1 h at 4°C. Microscopy was performed at 37°C using a Zeiss Axiovert wide-field microscope with a 100 X NA 1.4 plan-apochromat lens, a computer-controlled shutter and standard FITC/Alexa Fluor-594 filters. Images were collected with a CCD camera, with 2xbinning, delay times of 4-10 s and exposure times of 0.5-1 s/image. The collected images were further processed using Open Lab 2.2.5 software.

4.5 Infectivity titration, radioactive labeling and binding assay for EV1 (I, III, IV)

Confluent cells, infected with virus, were harvested after various time periods. After three freeze-thaw cycles, the amount of virus was determined by plaque assay in GMK cells. After an incubation of 7 days at 37°C, the cells were stained with crystal violet for the determination of end-point titers. For the plaque assay the viruses were incubated with the cells on 6-well plates for 30 min at 37°C. After removal of unbound virus, the cells were overlaid with 0.5% carboxymethylcellulose. The cells were incubated for 2 days prior to staining with crystal violet and counting the plaques.

To obtain radioactively labeled EV1, infected GMK cells were incubated with [^{35}S]methionine (50 μCi/ml, Pharmacia Biotech) in MEM without L-methionine (GibcoBRL, Life Technologies) and purified as described earlier (Abraham and Colonno, 1984).

For the binding assay, the cells were suspended into PBS containing 1-2 mM $MgCl_2$ and incubated with [^{35}S]methionine-labeled EV1 (75000 cpm) for 1 h on ice. The cells were washed and analyzed for radioactivity in a scintillation counter (1450 Microbeta; Wallac).

4.6 Electron microscopy (I, II, IV)

The cells were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide, dehydrated, stained with uranyl acetate, and embedded in LX-112. For visualizing clusters of α2 or αV integrin, cells were treated with anti-α2 or αV mAb, then with rabbit antibodies against mouse (Sigma) and finally with protein A gold (10-nm particles, G. Posthuma and J. Slot, Utrecht, The Netherlands). For visualizing EV1, the virus was attached on ice, incubated with EV1 antiserum and then with protein A gold. The samples were incubated at 37°C for different times.

For binding of cell lysate on grids, cells were incubated with primary and secondary antibodies as described above. After 30 min incubation at 37°C the cells were washed once, harvested in homogenizing buffer (3 mM imidazole, 0.25 M sucrose, 1 mM EDTA) and homogenized. The cell lysate was bound on grids for 40 min at room temperature and blocked with 10% FCS for 15 min. The samples were incubated with primary antibodies in 5% FCS/PBS for 40 min, washed, coupled with protein A gold (5-nm particles, G. Posthuma and J. Slot, Utrecht, The Netherlands) and washed. The samples were fixed in 2.5% glutaraldehyde for 5 min, washed in H_2O and coated with methylcellulose.

4.7 Integrin clusters and measurement of internalization (II, IV)

The cells were incubated with antibody against α2 integrin for 1 h on ice, washed, incubated with Alexa-conjugated secondary antibody on ice and washed. Formation of integrin clusters was allowed to occur at 37°C for 30-90 min. Clusters of αV integrins were performed in the same way, using anti-αV antibodies. As a control, anti-α2 and anti-αV mAbs, directly conjugated to Alexa 488, were used alone.

The internalization of EV1 or integrin clusters was estimated by confocal microscopy using the 3D for LSM program, version 1.4.2 (Carl Zeiss, Jena). Infected cells were fixed with 3% paraformaldehyde for 15 min and EV1 was stained without permeabilization using antiserum and then anti-rabbit Alexa 546-conjugate. After a 5 min permeabilization with 0.2% Triton X-100, EV1

was stained again but using the anti-rabbit Alexa 488-conjugate. Plasma membrane associated EV1 was stained with both Alexa 546 and Alexa 488 -conjugates and seen as yellow, whereas green signal represented internalized EV1. First the double stained cells were imaged as Z-stacks with confocal microscopy and then the volume of internalized vesicles and the total amount of fluorescence was measured using the 3D for LSM program. In a parallel experiment, integrin clusters were performed as described above using Alexa 488-conjugated anti-mouse antibody. The internalized integrins had green color and the plasma membrane associated integrins were labeled with Alexa 546-conjugate. Altogether, 20 cells were measured in both experiments.

4.8 SDS-PAGE and immunoblotting (I, II, IV)

The samples were separated in 10% SDS-polyacrylamide gel and electroblotted onto PVDF membrane (Millipore). Primary antibodies and horseradish peroxidase conjugated secondary antibodies (BIO-RAD) were used. Bands were detected by chemiluminescence (Pierce).

4.9 Protein kinase C activation (II)

Starved (overnight) cells were treated in different ways: 1) stimulated with phorbol 12-myristate 13-acetate (PMA, 1 μM) to activate PKC for 30 min at 37°C; 2) infected with EV1 for 30 min at 37°C; 3) pretreated with 10 μM safingol (Sigma) and followed by EV1 infection with 10 μM safingol for 30 min at 37°C; 4) incubated with anti-α2 Fab fragment (2 μg) for 1 h on ice, washed and incubated for 30 min at 37°C; 5) α2β1 integrin clustering as described above for 30 min at 37°C; 6) control cells were treated with medium. The cells were lysed with 30 mM octyl β-D-glucopyranoside (Sigma) for 30 min on ice.

4.10 Calpain activity (IV)

12-O-tetradecanoylphorbol-13-acetate (TPA, 100 ng/ml) was added to starved cells to activate calpains and incubated for 5 min. After that the levels of calpain 1 and 2 were determined by Western blot. For measuring calpain activity from detergent soluble and insoluble fractions, the incubation was continued for 5, 15 and 30 min, after which the cells were harvested and lysed in 0.1% Triton X-100 in Tris-HCl buffer. The lysates were centrifuged to separate detergent soluble and insoluble fractions and subsequently calpain activity was measured (TECAN Group. Ltd.).

4.11 Flotation gradient centrifugation (I-IV)

The cells were lysed with 1% Triton X-100 for 30 min on ice. The homogenate was adjusted to a sucrose density of 40.6% and overlaid with 5-35% sucrose. To study the uncoating of EV1, the samples were layered on a 5-20% sucrose gradient and centrifuged for 35,000 rpm at 4°C in a Beckman Sw 41Ti rotor for 18 h.

4.12 Immuno-isolation (I)

Infected cells were homogenized with a syringe and a needle in homogenizing buffer (3 mM imidazole, 0.25 M sucrose, 1 mM EDTA). The homogenate was pelleted and 100 μg of the supernatant was subjected to immuno-isolation. M-450 Dynal beads (2 x 10^7, Dynal AS) were coated overnight at 4°C with 3.75 μg of anti-caveolin-1 (clone 2234) or 3.75 μg of nonspecific mouse IgG (Sigma) together with 1% BSA. The beads were washed and incubated with the homogenates for 1 h by rotating at 4°C. The homogenate, a sample from the unbound fraction, and the beads were subjected to SDS-PAGE and immunoblotting.

To measure the infectivity of EV1 inside the caveolar stuctures the immuno-isolated material was treated with 0.5% SDS for 15 min on ice and the plaque assay was performed as described in 4.5.

4.13 Immunoperoxidase staining (I)

Cells in 24-well plates were incubated with antibodies: anti-α2 integrin, anti-β2 microglobulin and anti-HLA-1 or their combinations for 15 min. Cells were infected with EV1, and 10 h p.i. stained with EV1 antiserum and then with peroxidase-labeled goat anti-rabbit antibody (Dako). Then, the cells were stained with chromogen solution prepaired as described previously (Ziegler et al., 1988) containing H_2O_2.

4.14 Fluorescent *in situ* hybridization (III)

The negative-polarity RNA strand was transcribed using SP6 polymerase (Promega) from linearized EV1 cDNA (Ohman et al., 2001) in the presence of FITC-labeled UTP (Molecular Probes) or Chromatide Alexa Fluor 546-14-UTP (Molecular Probes). After DNase treatment, the probe was purified as described earlier (Bolten et al., 1998). Untransfected and GFP-caveolin-1-transfected cells were infected with EV1 and the hybridization reaction was

performed in the presence of the labeled probe for 12 h at 42°C in the dark. After removal of the unbound probe, the cells were mounted in 2.5% DABCO (Sigma) in pH-buffered glycerol and visualized using a confocal microscope (Leica).

4.15 Metabolic labeling and microinjection (IV)

Uninfected or EV1-infected cells in the presence or absence of 140 μM calpeptin were incubated in methionine/cysteine –deficient medium until 3 h p.i., after which [^{35}S]methionine (MP Biomedicals) label (100 μCi/ml) was added to the medium. The cells were scraped 8 h p.i. and incubated with 100 mM octyl β-D-glucopyranoside (Sigma) for 30 min on ice to effectively solubilize the membrane proteins. Labeled viral proteins were then analyzed by 10% SDS-polyacrylamide gel.

For microinjection cells on glass coverslips were washed and incubated in DMEM containing 1% FCS in the presence or absence of 140 μM calpeptin. Totally 50 cells were microinjected with EV1 RNA (isolated with High Pure RNA Viral Kit, Roche) and incubated at 37°C for 5 h.

4.16 *In vitro* translation (IV)

The reaction was performed according to the manufacturer´s instructions (Retic Lysate IV™ In Vitro Translation Kit, Ambion). The components of the reaction, including [^{S}35]methionine, were mixed in tubes and 12 μg of EV1 RNA (isolated with High Pure RNA Viral Kit, Roche) with or without 140 μM calpeptin were added on ice. The tubes were incubated at 30°C for 100 min in a water bath. The samples were incubated on ice for 5 min, subjected to 10% SDS-PAGE and analyzed by autoradiography.

5 REVIEW OF THE RESULTS

5.1 Entry of EV1

5.1.1 α2β1 integrin as a receptor for EV1

Investigation of EV1 uptake mechanism was started using SAOS-α2β1 cells (human osteosarcoma cells transfected with α2 integrin cDNA). When studied by confocal microscopy, EV1 was seen bound on the surface of SAOS-α2β1 cells 0 h p.i., and translocated to the perinuclear region by 2 h p.i. (I; Fig. 1A). The binding assay performed with radioactive EV1 confirmed that only 0.5% of virus was bound onto SAOS-pAW cells (control cells transfected with an empty vector), compared to over 70% binding onto SAOS-α2β1 cells (I; Fig. 2B). Only SAOS-α2β1 cells supported productive infection of EV1 (I; Fig. 1B, 2A, C).

To study if EV1 needed accessory receptors to initiate entry, specific antibodies were used to inhibit the infection. In addition to antibodies against α2β1 integrin which efficiently reduced the infection, two antibodies against β2 microglobulin blocked the infection almost totally (I; Fig. 2D). β2 microglobulin is known to be involved in the entry of EVs and related enteroviruses (Ward et al., 1998). As part of the HLA class I complex, the role of β2 microglobulin is to present foreign antigens to T cells. However, an antibody against HLA-1 had no apparent inhibitory effect on the infection (I; Fig. 2D).

Similar to infection in SAOS-α2β1 cells, EV1 caused rapid and efficient infection in CV-1 cells (III; Fig. 1B, C). These results are in line with previous observations showing that EV1 uses α2β1 integrin as a receptor (Bergelson et al., 1992; Bergelson et al., 1993b; Triantafilou and Triantafilou, 2001).

5.1.2 Interactions between EV1 and α2β1 integrin

For some picornaviruses, such as PV, the virus-receptor interaction has been described to lead to conformational changes in the virus that facilitates delivery of the viral RNA into the cytoplasm. Whether the attachment of EV1 to α2β1 integrin induced such alterations, was studied. After incubating radiolabeled EV1 with α2β1 integrin on ice for 1 h, no empty capsids sedimenting in the 80S form were detected (I; Fig. 3A, III; Fig. 8A). Thus, EV1 binding to its receptor seemed not to trigger the uncoating process.

It was of interest to see whether α2β1 integrin acted as an active receptor guiding the virus to a specific endocytic route. Interestingly, confocal microscopy revealed that after colocalizing with EV1 on the plasma membrane the receptor also internalized with the virus. The redistribution of virus-receptor complex and its accumulation intracellularly increased in a linear manner during the first hours of infection, and at 2 h p.i. 70% of the cells showed perinuclear colocalization of EV1 and α2β1 integrin (I; Fig. 4A). Although α2β1 integrin and β2 microglobulin exhibited some colocalization on the cell surface following EV1 binding (not shown), β2 microglobulin did not colocalize significantly with α2β1 integrin in the perinuclear region (I; Fig. 4B).

Flotation gradient centrifugation indicated that ligand-free α2β1 integrin was found in detergent resistant membrane fractions together with GPI-APs and caveolin-1 (II; Fig. 1A). Alexa 546-conjugated mutant aerolysin (ASSP), which binds to the GPI moiety of GPI-APs, was used in studies. Aerolysin is a bacterial toxin, which upon insertion into a lipid bilayer forms a pore. The mutant ASSP remains inactive but can be used to detect the GPI-APs (Fivaz et al., 2002). Confocal microscopy was needed to reveal that α2β1 colocalized on the cell surface with ASSP, thus localizing to GPI-AP enriched domains, rather than to caveolae (II; Fig. 1B).

Receptor clustering was initiated using primary anti-α2 integrin antibodies followed by secondary antibodies. The clustered integrins started to redistribute out of GPI-AP enriched domains (II; Fig. 2A). They simultaneously fused together to form larger clusters, which was visualized by live cell confocal microscopy (II; Fig. 2B). The integrins seemed to traffic along cortical actin microfilaments (II; Fig. 2C, D, E). In CV-1 cells, EV1 infection was unaffected by cytochalasin D (an inhibitor of actin polymerization) treatment (III; Fig. 6C). Testing of two other agents interfering with actin function, latrunculin (an actin monomer-sequestering drug) and jasplakinolide (an actin polymer-stabilizing drug) gave similar results in CV-1 cells (III; Fig. 6C). After the translocation step, integrin clusters were internalized efficiently into the cytoplasm (71 ± 3% of α2β1 integrin being intracellular at 2 h) (II; Fig. 3A). No clustering or internalization of integrin was observed if cells were treated with fluorophore-labeled primary antibody without secondary antibody (II; Fig. 3B). This data indicated that formation of α2β1 integrin clusters is sufficient to induce internalization of the receptors, and EV1 as a multivalent ligand can trigger the same endocytic process as the antibodies.

5.1.3 Entry into caveosomes

Since many viruses, such as HPEV1 (Joki-Korpela et al., 2001), are known to use the clathrin-dependent endocytic route in cell entry, the possible connection of EV1 and integrin to the clathrin route was tested. EV1 or α2β1 integrin did not colocalize with protein markers for early, recycling or late endosomes, nor with the Golgi complex (I; Fig. 5). Electron micrographs indicated that the surface of SAOS-α2β1 cells was rich in caveolae (I; Fig. 6A), and EV1 could be seen 30 min to 2 h p.i. in the intracellular vesicles, which tended to accumulate close to the nucleus (I; Fig. 6 B-D). The vesicles had no visible coat and they were positive for caveolin-1 (I; Fig. 6 E, F). In order to confirm the role of caveolin-1 in EV1 infection, caveolar structures were immuno-isolated with magnetic beads coated with anti-caveolin-1, which recognizes the intact vesicles (Oh and Schnitzer, 1999). Larger amounts of virus could be isolated with immunobeads 15 min p.i. than after virus binding on cells (0 h p.i.) (I; Fig. 8). Correspondingly, the amount of isolated α2β1 integrin was greater after internalization for 15 min (not shown). Behaving like EV1, clustered integrins internalized into similar vesicles (II; Fig. 3 E, F).

In confocal images some caveolin-1 staining on the plasma membrane was seen (II; Fig. 1B), but between 30 min and 2 h p.i. caveolin-1 showed considerable perinuclear staining and colocalization with α2β1 integrin and EV1 (I; Fig. 7). Again, the antibody-induced integrin clusters trafficked in a similar manner as EV1 into caveolin-1 positive perinuclear accumulations (II; Fig. 3 C, D), resembling caveosomes (Pelkmans et al., 2001). In CV-1 cells confocal microscopy indicated a similar internalization process (III; 1A).

To exclude the clathrin-dependent processes in the clustered α2β1 integrin entry, transfections with Eps15 and AP180 mutants were performed. The role of Eps15 is to dock AP-2 complex on the plasma membrane during the CCP assembly (Benmerah et al., 1999), whereas AP180 tethers clathrin to the membrane (Ford et al., 2001). Although the mutants were effective inhibitors of transferrin entry, the internalization of integrin was unaffected (II; Table 1). The Eps15 mutant failed to inhibit EV1 infection in CV-1 cells also (III; Fig. 6A). Furthermore, when αV integrins were clustered with antibodies, they were internalized in caveolin-1 negative vesicles (II; Fig. 4 A). Some of the vesicles containing αV clusters were positive for EEA-1 and endocytosed transferrin (II; Fig. 4 B, C), in contrast to α2β1 integrin containing vesicles, which were negative for EEA-1 and transferrin (II; Fig. 4 B, C). Electron micrographs confirmed the presence of clathrin coat on the αV integrin containing vesicles (II; Fig. 4 D).

The kinetics of EV1 trafficking was followed in GFP-tagged caveolin-1 –expressing CV-1 cells by real-time fluorescence microscopy using Alexa Fluor (AF) –labeled EV1. Now the first stages of the entry were investigated also. The majority of bound EV1 did not colocalize with caveolin-1 on the cell surface, and only after 10 min the virus moved into mobile structures positive for caveolin-1. During the first 20 minutes the colocalization of caveolin-1 and EV1 increased in organelles, which performed movements of different distances (III; Fig. 2E, 3A). The size and number of internalized caveolin-1 positive vesicles increased over time, leading to the formation of larger accumulations in the cell periphery and perinuclear regions. In the vesicles EV1 partially colocalized with cholera toxin B (CTxB) 30

min to 1 h p.i. CTxB is known to partially pass through caveosomes during transport to the Golgi complex (Nichols, 2002) (III; Fig. 4A). Afterwards, EV1 did not colocalize with Golgi markers (III; Fig. 4B). EV1 also colocalized to some extent with SV40, a well-known representative of the caveolar route and caveosomes (Pelkmans et al., 2001). Co-infections of SV40 and EV1 were synchronized by incubating SV40 for 90 min before binding of EV1, because EV1 entry is much faster. Confocal microscopy showed partial colocalization at 30 min after EV1 entry, which increased during the next 2 h (III; Fig. 5A). In live microscopy, colocalization of SV40 and EV1 was observed 15 min after EV1 entry (III; Fig. 5B). Again, the size and mobility of the structures positive for SV40 and EV1 increased with time. Moreover, nocodazole treatment, which prevents trafficking of SV40 to the smooth ER (Pelkmans et al., 2001), increased the colocalization of SV40 with EV1 in the caveolin-1 positive structures (III; Fig. 5C). Taken together, the presence of caveolin-1, CTxB and SV40 with EV1 in the same intracellular structures devoid of markers of the clathrin route, suggested that EV1 entered caveosomes.

5.1.4 Role of caveolin-1

Despite the fact that association of EV1 with caveolin-1 was not obvious in the first minutes of infection, caveolin-1 was apparently needed later in the EV1 life cycle. Previous studies have shown that integrins can be coprecipitated with caveolin-1 (Wary et al., 1996; Wary et al., 1998), and caveolin-1 is a regulator of β1 integrin function (Wei et al., 1999). Expression of truncated and dominant negative caveolin-3 (cav^{DGV}), which disrupts lipid transport and depletes cell surface cholesterol (Roy et al., 1999; Pol et al., 2001), caused 66% inhibition of infection in SAOS-α2β1 cells (I; Table 1) and 35% inhibition of infection in CV-1 cells (III; Fig. 6A).

Since cholesterol is a central component of GPI-AP enriched domains and caveolae, the effect of disturbing cholesterol homeostasis was investigated. Treatment with methyl β-cyclodextrin (MBCD), which affects cholesterol distribution and destroys caveolae decreased EV1 infection from 63% to 9% in SAOS-α2β1 cells (I; Table 2). In addition, it reduced the amount of virus capsid proteins internalized into the perinuclear region and the number of cells showing perinuclear colocalization of EV1, caveolin-1 and α2β1 integrin (I; Table 2). In CV-1 cells, MBCD treatment inhibited EV1 infection almost completely, by 95% (III; Fig. 6B). In CV-1 cells a combination of progesterone and nystatin was tested also, showing inhibition of EV1 infection by more than 50% (III; Fig. 6B). Progesterone inhibits cholesterol synthesis and nystatin is a cholesterol sequestering drug. Although the cellular effects of MBCD may be extensive, these results implicated the role of cholesterol and caveolin-1 in the EV1 replication cycle.

5.2 EV1 entry-related signaling

Formation of integrin clusters is important for signaling events (Miyamoto et al., 1995). Furthermore, MAPKs have been shown to be regulated by the collagen

receptor integrins (Ravanti et al., 1999). Here, extracellular signal regulated kinase (ERK) was activated 15 min after α2β1 integrin binding to EV1 in SAOS-α2β1 cells, and the expression of phosphorylated ERK clearly diminished after 1 h p.i. (II; Fig. 5A). Next, candidates of the same pathway, upstream of ERK, were screened for their participation in the signaling by EV1 trafficking (II; Fig. 5B, C). ERK activation was judged by accumulation in the nucleus and it was inhibited by two PKC inhibitors, bisindolylmaleimide (a pan inhibitor) and safingol (specific for the α isoform of PKC) (II; Fig. 5D). Both inhibitors reduced the amount of EV1 infected cells to the background level (II; Fig. 6A) and the dominant negative construct of PKCα inhibited EV1 infection by 50% (II; Fig. 6B). Chronic phorbol ester (PMA) treatment was used to down-regulate PKCs and, as a result, EV1 infection was prevented (II; Fig. 6C). PKCα obviously is a resident and constantly active protein in caveolae (Smart et al., 1994; Mineo et al., 1998). Accordingly, untreated SAOS-α2β1 cells contained rather high levels of phosphorylated PKCα and PMA could only slightly increase its phosphorylation. Similar small increase in the phosphorylation of PKCα was seen after the cells were exposed to EV1 or to antibodies forming α2β1 integrin clusters (II; Fig. 6D).

PKCα directly binds to the cytoplasmic domain of integrin β1 subunit thereby regulating the internalization of the receptor (Ng et al., 1999a). Here, PKCα activity was needed for the internalization of EV1 and integrin (II; Fig. 7C, D, E), but not for internalization of clustered αV integrin (II; Fig. 7E). The action of PKCα was limited to the first minutes of the process (II; Fig. 7A), after binding of EV1 to the integrin receptor (II; Fig. 7B). The α2/α1β1 integrin mutant containing the intracellular tail of the α1 integrin subunit, had no inhibitory effect on the production of infectious EV1. The result confirms the role of β1 integrin in the EV1 internalization (I; Fig. 2E).

In CV-1 cells PKCα activity was similarly crucial for EV1 infection (III; Fig. 6B). Tyrosine kinase activation seemed to be important for the EV1 replication cycle. Genistein, a tyrosine kinase inhibitor known to inhibit caveolar uptake of autocrine motility factor (AMF), cholera toxin and SV40 (Le and Nabi, 2003), blocked the infection in a dose-dependent manner. However, okadaic acid, a general inhibitor of serine and threonine phosphatases, which increases caveolar internalization (Parton et al., 1994), had no significant effect on EV1 infection. Sodium orthovanadate (NaOV), an inhibitor of protein tyrosine phosphatases enhancing the uptake of SV40 via caveolae (Pelkmans et al., 2002), reduced EV1 infection by 70% (III; Fig. 6B). Dynamin 2 is a GTPase acting in both the clathrin-dependent and the caveolar route (Henley et al., 1998; Oh et al., 1998). The expression of a dominant negative mutant of dynamin 2 (K44A) inhibited EV1 infection by 75% (III; Fig. 6A) in CV-1 cells.

5.3 Indications of uncoating and replication

5.3.1 Uncoating

Incubation of radioactively labeled EV1 with SAOS-α2β1 or GMK cells for 30 min to 2 h resulted in the appearance of 80S empty capsids, indicating that uncoating occurs after endocytosis (I; Fig. 3B, III; Fig. 8B). Supporting this, immuno-isolated caveolar structures contained infective virus (I, p. 1863).

Caveosomes obviously represent the last cellular organelle containing EV1 particles, since their localization did not change at later time points (III; Fig. 4B). In contrast to SV40, which continues from the caveosomes to the smooth ER in a microtubule-dependent manner (Pelkmans et al., 2001), EV1 did not traffic to the smooth or rough ER (III; Fig. 4B). EV1 capsid proteins colocalized substantially with the viral RNA 30 min to 1 h p.i., when FISH technique (Bolten et al., 1998) was used in combination with the detection of AF-EV1 in CV-1 cells (III; Fig. 9A). The colocalization was clearly diminished 2 h p.i., suggesting release of viral RNA from the capsids. Viral RNA positive structures colocalized with GFP-caveolin-1; again, not remarkably in the beginning of the entry, but becoming more evident 30 min to 1 h p.i. (III; Fig. 9B). This indicated that the sites of EV1 decapsidation are caveosomes.

5.3.2 Start of replication

A considerable increase in the amount of viral RNA staining was seen by FISH technique 4 h p.i. in the cytoplasm of CV-1 cells (III; Fig. 8C). The observation is consistent with the start of production of infectious EV1 in SAOS-α2β1 cells 2.5-3.5 h p.i. (I; Fig. 2E). Newly synthesized viral capsid proteins were seen to fill the cytoplasm of SAOS-α2β1 cells 4 h p.i. (IV; Fig. 1A), and Western blot analysis showed that the expression of EV1 3D RNA polymerase increased 4 h p.i. (IV; Fig. 1B). Monitoring the synthesis of negative and positive strands of EV1 RNA by quantitative RT-PCR showed that the copy number of both viral RNA strands increased between 3 and 4 h p.i. After 4 h p.i., the increase was very fast (IV; Fig. 6B-E).

Treatment of SAOS-α2β1 and CV-1 cells with brefeldin A (BFA), a Golgi-disrupting agent (Dinter and Berger, 1998), caused EV1 to accumulate in large caveosomes and inhibited replication of viral RNA, since newly synthesized RNA did not appear in the cytoplasm.

5.4 Role of calpains in the EV1 replication cycle

5.4.1 The effect of calpain inhibition on EV1 infection

Since calpains can degrade cytoplasmic domains of integrins (Pfaff et al., 1999), and integrins have a guiding role in EV1 infection, studies of the possible

involvement of calpains in EV1 infection were initiated. Calpeptin, an inhibitor of calpains 1 and 2, caused a total block of EV1 infection not only in SAOS-α2β1 cells (IV; Fig. 6A, 1D), but also in 293 and HeLa MZ cells (IV; Fig. 2C). Confocal microscopy revealed that in both control and calpeptin-treated cells, EV1 capsid proteins were found in the perinuclearly accumulated vesicles 2 h p.i. However, in calpeptin-treated cells, EV1 remained in the vesicles up to 8 h p.i. with no capsid protein synthesis taking place (IV; Fig. 1A). As an indication of EV1 protein synthesis, the expression of 3D RNA polymerase started 4 h p.i. (IV; Fig. 1B) which was not observed in the cells treated with calpeptin (IV; Fig. 1C). Additionally, calpain inhibitors 1 and 2 effectively blocked the infection (IV; Fig. 1F). Participation of calpains 1 and 2 in the EV1 replication cycle was confirmed using specific siRNAs for the calpains (IV; Fig. 1E). They downregulated calpain 1 and calpain 2 expression at mRNA level approximately 85% (not shown). An earlier study has shown that several protease inhibitors interfere with the proteolytic activity of PV $2A^{pro}$ (Molla et al., 1993). However, when selected protease inhibitors were tested, they had no significant effects on EV1 life cycle (IV; Fig. 1G, 6F). Thus, the described effect was not due to unspecific inhibition of viral proteases.

Involvement of calpains was tested in infection by two other picornaviruses, HPEV1 and CVB3 which both utilize clathrin-dependent endocytosis (Joki-Korpela et al., 2001; Chung et al., 2005). Calpeptin inhibited HPEV1 infection by 54% in A549 cells (IV; Fig. 2A) and specific siRNAs for calpain 1 and calpain 2 confirmed the role of calpains in the infection (IV; Fig. 2B). The infection by CVB3, a virus belonging to the same *Enterovirus* genus as EV1, was prevented completely by calpeptin treatment in SAOS-α2β1 and GMK cells (IV; Fig. 2C). Thus, calpains seem to be of importance also for other single-stranded RNA viruses, especially those of the same genus.

5.4.2 Increase in calpain activity following EV1 entry

In confocal microscopy images, both calpain 1 and calpain 2 localized partially to plasma membrane domains positive for GPI-APs (detected with aerolysin, ASSP) (IV; Fig. 5A). When the cells were extracted with Triton X-100, calpain 1 was found mostly in the detergent soluble fraction, whereas calpain 2 was also present in the detergent insoluble fraction (IV; Fig. 5B), as has been reported previously (Morford et al., 2002; Hood et al., 2003; Kifor et al., 2003). After EV1 internalization, drastic recruitment of calpain 1 and calpain 2 to the virus containing vesicles was seen by confocal microscopy (IV; Fig. 5C) and immuno electron microscopy (IV; Fig. 5D, only calpain 2 is shown).

Activity assays were used to study calpain activity stimulated by EV1. Testing with phorbol ester (PMA) caused a remarkable increase in calpain activity, which was seen after 5 min in the detergent insoluble fraction and after 15 min in the detergent soluble fraction (IV; Fig. 3A). EV1 infection could increase calpain activity 1.4 fold in the detergent soluble fraction 3 h p.i. (IV; Fig. 3B, C), whereas no increase was seen in the expression levels of calpain 1 or 2 (IV; Fig. 3D).

5.4.3 Importance of calpains in the replication of EV1

Calpeptin had no effect on the number of EV1 particles or α2β1 integrin clusters internalized (IV; Fig. 4A). When calpeptin was added at different time points after EV1, it could cause a total inhibition; even when added 2.5 h p.i. (IV; Fig. 4B). Similar results were seen with calpain inhibitor 1 and calpain inhibitor 2 (IV; Fig. 4C), clearly indicating the role of calpains at later stages of EV1 replication cycle.

When EV1 genomic RNA was microinjected into SAOS-α2β1 cells in the presence of calpeptin, the infection was prevented (IV; Fig. 6A). Calpeptin had no effect on *in vitro* translation of EV1 RNA in the rabbit reticulocyte system (IV; Fig. 6G), but calpeptin and calpain inhibitors 1 and 2 (IV; Fig. 6B-E) prevented the synthesis of negative and positive strands of EV1 RNA, as quantitated with RT-PCR.

6 DISCUSSION

As the principal cell surface adhesion receptors, integrins have irreplaceable cellular functions. Signals transmitted by them determine the fate of cells. EV1, a picornavirus, takes advantage of the physiological function of its receptor, α2β1 integrin and gains entry into the cell. Molecular pathogenesis of picornavirus infections is poorly understood. The receptor may contribute to the pathogenesis by guiding the virus to a specific entry route. Here, the life cycle of EV1 from its receptor binding to the beginning of replication was followed. The productive EV1 infection was shown to depend on the internalization of α2β1 integrin, signaling events, localization to caveosomes and finally, activity of calpain proteases in the initiation of the replication.

6.1 EV1 entry

6.1.1 EV1-receptor interactions

EV1 is the only picornavirus using α2β1 integrin as a receptor that probably has many implications in the entry and pathogenesis of the virus. The question, how a particular receptor is selected, arises. Typical to cell adhesion receptors, such as integrins, is their low binding affinity to physiological ligands (Wang, 2002). Viruses, in contrast, have evolved a stronger binding affinity to compete with the weaker natural ligands. Picornaviruses with icosahedral structure may mimic the multivalent ECM proteins and, as a result of multiple interactions with the receptor, attach firmly. For EV1, it has been shown that the integrin binding site, the α2I domain, is structurally more globular than the elongated domains of ICAM-1 and PVR. Consequently, the α2I domain contributes to more extensive contacts with the virus canyon wall (Xing et al., 2004).

There are other viruses that utilize α2β1 integrin in the entry, such as HCMV and rotavirus. However, they can also use other integrin heterodimers as their receptors (Graham et al., 2003; Feire et al., 2004). In this study, the previous findings were confirmed by showing that EV1 infection is prevented with anti-α2

integrin (Bergelson et al. 1992) and anti-β2 microglobulin antibodies (Ward et al. 1998). It is not known if β2 microglobulin acts as a coreceptor or has some indirect role in EV1 entry. One possibility is that it interferes with the aggregation of integrins on the membrane and inhibits the productive entry of EV1.

Conformational alterations in the virion structure are essential for genome release of picornaviruses. Binding of PVs and major group HRVs to their receptors induces rearrangement of virus capsid proteins that may lead to a release of the RNA genome across the plasma membrane (Hoover-Litty and Greve, 1993; Tosteson and Chow, 1997). In this study, the interaction of EV1 with α2β1 integrin did not induce formation of empty virus capsids (I, III). The results were confirmed later (Xing et al., 2004). However, some EV1 particles showed conversion from the native 160S form to the approximate 135S form shortly after binding to the cell surface. The 135S particles of EV1 may not be similar to those of PV1 and more investigation is needed to reveal the nature of the altered EV1 particles.

Integrins have been shown to localize to discrete plasma membrane GPI-AP enriched domains, which have a high concetration of cholesterol (Thorne et al., 2000; Claas et al., 2001). These domains may contribute to the conversion of integrins to a high-affinity state (Wei et al., 1999; Leitinger and Hogg, 2002; Decker and ffrench-Constant, 2004). On the other hand, integrin-mediated adhesion seems to be essential for the integrity and maintenance of GPI-AP enriched domains, since cell detachment can quickly trigger internalization of proteins localizing to these regions (del Pozo et al, 2004). In focal adhesions, where clustered integrins link the actin cytoskeleton to the ECM, particularly stable GPI-AP enriched domains have been observed (Gaus et al., 2006). In this study, ligand-free α2β1 integrin was seen to reside in GPI-AP enriched domains. When anti-α2 integrin antibodies were used to induce cluster formation, integrins began to move laterally and subsequently internalized (II). The movement along the cortical actin filaments seemed to depend on the polymerization of actin, since treatment with cytochalasin D, which inhibits the polymerization, inhibited the locomotion of α2β1 integrins. Recently, it has been reported that different conformational states of integrins exhibit different rates of mobility and degrees of cytoskeletal attachment. Integrin conformations are recognized by cytoskeletally attached proteins, which can control lateral movement and clustering of integrins (Cairo et al., 2006). Possibly, the occupation by a multivalent ligand releases integrins from the actin stress fibers at the site of adhesion, but the subsequent translocation step requires intact cortical actin filaments. EV1, behaving as a multivalent ligand (Xing et al., 2004), activated the same internalization process of α2β1 integrins as antibodies and gained entry into cells.

Interaction of EV1 with its receptor appeared to be long-lasting. After following the virus into the cells, α2β1 integrin remained in close connection with EV1 during the observed infection periods. The number of studies addressing endocytosis of integrins is relatively low. Conventionally, endocytosis of cell surface receptors is thought to downregulate the receptors and attenuate their signaling capabilities. Maybe in EV1 infection, the role of α2β1 integrin is to act as an intracellular guide, or a shelter of the virus. Alternatively, after the binding events on the cell surface, integrin can continue signaling intracellularly to promote EV1 replication.

6.1.2 Internalization into caveosomes

Endocytosis of most viruses is mediated through the clathrin-dependent entry route. As an example, uptake of HRV2 into acidified vesicles of the clathrin–mediated route is obligatory for the release of RNA (Snyers et al., 2003). EV1, like PV, is an acid stable virus and does not necessarily need low pH for uncoating (Perez and Carrasco, 1993). Accordingly here, no evidence of EV1 internalizing in the clathrin-coated vesicles was observed. Instead, α2β1 integrin clustered by EV1 or antibodies, entered caveosomes, which differ from the classical endosomal vesicles by being neutral and lacking markers of the endosomes (Pelkmans et al., 2001) (I, II, III). Trafficking by caveolae is less well understood than by e.g. the CCPs. Compared to the clathrin-dependent entry route, endocytosis via caveolae is slower. It takes 20 min for SV40, a model for the caveolar pathway, to internalize from the plasma membrane, and additional 20 to 40 min to reach the caveosomes (Pelkmans et al., 2001). For EV1, in contrast, it took approximately 15 min to enter caveosomes, suggesting the use of another internalization route. Furthermore, the dominant negative mutant of caveolin-1 (GFP-caveolin-1), which inhibits the entry and infection of SV40 (Pelkmans et al., 2001), had no blocking effect on EV1 infection (V. Pietiäinen, unpublished). Focusing on the first minutes of the infection indicated that colocalization of EV1 with caveolin-1 was not obvious on the cell surface, but increased gradually with time. Since the routes of SV40 and EV1 merged at the level of caveosomes (III), the EV1-containing vesicles obviously fused with them.

Evidently, there is crosstalk and fusion between the different endocytic systems that provide options for viruses to exploit. Usually, clathrin- and caveolar–independent carrier vesicles have been shown to fuse with endosomes (Kirkham and Parton, 2005). Evidence of fusion with caveosomes is lacking. GPI-APs have been reported to internalize independent of clathrin or caveolin into GPI-AP enriched early endosomal compartments (GEECs). From there they are sorted to different endosomal destinations, depending on the cell type (Sabharanjak & Sharma, 2002; Kalia & Kumari, 2006). SV40 is also capable of using another, caveolar-independent entry route, which targets the virus-containing vesicles to structures resembling caveosomes, but negative for caveolin (Damm et al., 2005). Fusion of EV1 carrier vesicles to caveosomes may imply a means, which assures the correct uncoating of the virus. Possibly the biochemical content of the membranes or the molecular composition of caveosomes are of importance for EV1 life cycle.

The role of caveolin-1 in EV1 infection is not perfectly clear. Expression of cav^{DGV}, which inhibited EV1 infection partly in two host cell lines (I, III), evidently interferes with cellular functions through aberrations in cholesterol homeostasis. Cholesterol depletion and lipid composition of the plasma membrane may affect the cell adhesion and signaling via β1 integrins. Namely, addition of cholesterol stimulates clustering of β1 integrins and hence the avidity to ligands (Sharma and Brown 05). Presumably, depletion of cholesterol acts in the opposite way, impeding internalization and signaling of integrins. This may explain the preference of α2β1 integrin for GPI-AP enriched domains and the almost complete inhibition of EV1 infection upon extraction of cholesterol (I, III).

During this work, the concept of EV1 uptake into the host cells has evolved. In the beginning, the early steps of the internalization were not investigated. The assumption that EV1 internalizes via caveolae was based on the immunofluorescence and electron microscopy data of the later timepoints. Also, the results of the immuno-isolation study suggested that EV1 entry utilized the actual caveolar structures. Although, after binding of EV1 to the receptor a fraction of viral proteins could be isolated with anti-caveolin-1 beads, the amount of EV1 in caveolar structures was remarkably higher 15 min p.i. (I). Only recently, the first minutes of the EV1 entry have been studied in detail (III). The results strongly suggest that the primary endocytic route of EV1 is independent of caveolae, although some EV1 particles might use the caveolar pathway also.

6.2 EV1 entry-related signaling

MAPK signaling has been implicated in CVB3 pathogenesis. The virus activates ERK in two phases, first transiently right after the infection and later, sustained activation is seen towards the final stages of the replication. Also, the activity of p38 MAPK is required for CVB3 viral progeny release (Wong et al., 2007). Correspondingly, in this study, ERK was phosphorylated transiently 15 min p.i (II). In the later stages of EVI infection, activation of both the growth-related ERK1/2 (5 h p.i.) and stress-activated p38 MAPK pathways (10 h p.i.) have been reported (Huttunen et al., 1997; Huttunen et al., 1998). The events, in which EV1-related ERK activation is required, need more investigations. The activation of EV1-related p38 MAPK could be linked to cell lysis and virus release, since the infection cycle of EV1 is approximately 10 h (Pietiäinen et al., 2000).

Caveolar and clathrin –dependent endocytic routes, as well as some routes independent of caveolin and clathrin, require dynamin (Marsh and Helenius, 2006). Here, the mutant dynamin 2 (K44A) remarkably inhibited EV1 infection in CV-1 cells (III). EV1 entry in SAOS-α2β1 cells, on the contrary, seems not to depend on dynamin (M. Karjalainen and E. Kakkonen, unpublished). The preliminary results suggest that EV1 entry may be regulated by the carboxy-terminal binding protein/Brefeldin A-ribosylated substrate (CtBP/BARS) (P. Liberali, unpublished), which is implicated in the dynamin-independent endocytosis (Bonazzi et al., 2005). Caveolar-mediated internalization of SV40 requires a transient depolymerization of cortical actin, leading formation of actin tails (Pelkmans et al. 2002). In this study, EV1 entry in CV-1 cells was not prevented by actin inhibitors (III). In contrast, for EV1 endocytosis in SAOS-α2β1 cells, a short depolymerization of actin seems to be essential, since the actin stabilizing drug jasplakinolide has been shown to inhibit the infection (M. Karjalainen and E. Kakkonen, unpublished). The opposite results in the two host cells can likely be explained by different endocytic mechanisms in general.

PKCα can interact directly with the cytoplasmic domains of β1 integrins, thereby regulating the internalization of integrins (Ng et al., 1999a). The activation mechanisms of β1 integrin and PKCα seem to be mutual, as TPA-activated PKCα is able to induce clustering and concomitant activation of unligated α2β1 integrin

(Connors et al., 2007). In this study, PKCα regulated the internalization of EV1 and α2β1 integrin via the β1 integrin tails and consequently, was required for the productive infection of EV1 (II, III). The caveolar-mediated entry of SV40 is regulated by tyrosine kinases and enhanced with inhibitors of protein tyrosine phosphatases (Pelkmans et al., 2002). Also the caveolar-independent entry of SV40 requires the activity of tyrosine kinases (Damm et al., 2005). Here, EV1 infection was dependent on both tyrosine kinase and tyrosine phosphatase activities (III), convincing the usage of another entry route. Possibly the initial step of EV1 entry is dependent on the tyrosine kinase activity, whereas tyrosine phosphatases are important after that, in e.g. the actin rearrangements. Namely, in PV infection, tyrosine phosphatases are suggested to participate in the actin remodeling during the virus entry (Coyne et al., 2007).

6.2.1 EV1 uncoating and replication

Picornavirus uncoating involves alterations in the virus particle, loss of structural proteins and a penetration mechanism to release the RNA genome into cytoplasm for replication. The genome passes either through the plasma membrane or a limiting membrane of an endocytic organelle. Regardless of years´ research on PV1, the entry mechanism of the virus remains unclear. The newest data demonstrate that PVR follows the virus into the cell via caveolae and remains in proximity with PV up to 3 h p.i. Conversion of PV capsid into the 135S particles occurs rapidly on the cell surface, but the site of RNA release remains unknown (Coyne et al., 2007).

Caveosomes obviously are the site of EV1 uncoating since EV1 did not move to any other cellular locations from there. How EV1 RNA is released from caveosomes, is currently under investigation. It might occur through a pore or a rupture in the organelle membrane but the trigger for that is unknown. By using the novel electron microscopic tomography methods, the structural details of caveosomal membranes and internalized cargo can be examined and the possible alterations in the membranes detected.

Picornavirus replication takes place on the cytosolic surface of double membraned vesicles, replication complexes. Different viruses utilize distinct mechanisms to assemble their replication complexes. The origin of PV-induced vesicles has been under debate. It has been suggested that PV utilizes the secretory route in the biogenesis of replication complexes by a Brefeldin A (BFA)-sensitive manner (Belov and Ehrenfeld, 2007; Belov et al., 2007). BFA is a fungal metabolite, which inhibits vesicle transport by dissociating of membrane-associated budding machinery, including ARF (Mossessova et al., 2003). Alternatively, PV and HRV may induce the formation of autophagosome-like double-membraned vesicles, on which their replication occurs. Fusion of the outer autophagosomal membrane with the plasma membrane would provide a mechanism for viral release without cell lysis (Jackson et al., 2005). Defining the replication complex of EV1 requires more studies. Possibly the ARF-dependent reactions are also regulating some steps of EV1 life cycle, because BFA treatment resulted in accumulation of EV1 into large caveosomes and inhibition of infection (III). The possibility, that EV1 life cycle involves a nonlytic viral escape, would be interesting to study as well.

6.3 Calpains in the EV1 replication cycle (IV)

Calpains are abundant intracellular proteases dependent on Ca^{2+}. They play an important role in integrin-related cytoskeletal reorganizations by regulating the activity e.g. FAK and PKC (Carragher et al., 1999; Kishimoto et al., 1989) and cleaving the cytoplasmic domains of β1 integrins (Pfaff et al., 1999). Several viral infections are known to evoke activation of calpains (Molla et al., 1993; Debiasi et al., 1999; Chen et al., 2001; Teranishi et al., 2003; Kalamvoki and Mavromara, 2004). Interestingly, calpains participated in the life cycle of EV1 also. Inhibition of calpains blocked EV1 infection but had no effect on the internalization of EV1 or α2β1 integrin. The surmise was that calpain activity was not needed in the rearrangement of actin cytoskeleton during the viral entry. Calpains 1 and 2 were able to degrade α2β1 integrin *in vitro* (L. Nissinen, unpublished) and they accumulated into EV1 and α2β1 integrin containing caveosomes. Therefore, the possibility, that calpains would destroy the structure of caveosomes in order to release EV1 RNA, was examined. It is not known if the caveosomal membranes contain natural targets of calpain-mediated proteolysis. However, neither caveolin-1 nor EV1 capsid proteins were sensitive to calpains (P. Upla, unpublished). Another possibility was, that by degrading α2β1 integrin, calpains would dissociate the stabilizing α2I domain from EV1, and induce uncoating. To see if calpain inhibition had any effect on the viral RNA release, confocal studies with molecular beacons recognizing EV1 RNA, as well as sucrose gradients with radioactive EV1, were performed (P. Upla, unpublished). Calpeptin, an inhibitor of calpains 1 and 2 were used in the studies. Experiments with the molecular beacons indicated that structures containing EV1 RNA were not formed in the presence of calpeptin but whether the drug inhibited EV1 RNA release or formation of replication complexes, is currently difficult to state. Calpeptin treatment also had some inhibitory effects on the formation of EV1 empty capsids but did not totally inhibit it. Although no full certainty was gained, involvement of calpains in uncoating and/or release from caveosomes cannot totally be ruled out. Therefore, working on the EV1 uncoating is ongoing.

The process, by which calpains become activated, is not completely understood. It is believed to occur primarily in association with cellular membranes and with a definite requirement for Ca^{2+} (Goll et al., 2003). EV1 infection led to an increase in the cytosolic Ca^{2+} concentration, which likely contributed to calpain activation (M. Karjalainen, unpublished). Of picornaviruses, PV and CVB3 infections have been shown to cause a notable increase in the intracellular Ca^{2+} level. Their viral proteins are capable to enhance membrane permeability and influx of Ca^{2+} from the ER and extracellular sources (Irurzun et al., 1995; van Kuppeveld et al., 1997). It is possible that the membrane-active viral proteins have a role also in EV1 replication cycle, leading to the elevated Ca^{2+} concentration 1 to 4 h p.i. Calpains were evidently critical in the later stages of EV1 life cycle. What constitutes the upstream and downstream components of a signaling cascade, within which calpains are involved, can only be speculated. Calpeptin treatment could not prevent the *in vitro* translation of EV1 RNA using rabbit reticulocyte system but it blocked the replication after microinjection of EV1

genomic RNA into the host cells. The role of calpains could be to regulate the activity of the viral proteins or formation of replication complexes.

An earlier study has shown that several protease inhibitors, including calpain inhibitor I, could inhibit the proteolytic activity of PV $2A^{pro}$ (Molla et al., 1993). Here, selected protease inhibitors had no effect on EV1 infection. Importantly, downregulation of calpains 1 and 2 with siRNAs blocked EV1 infection, confirming that the described effect was not due to unspecific inhibition of viral proteases. Finally, calpeptin could inhibit infection by two other picornaviruses, HPEV1 and CVB3. Interestingly, many viruses seem to depend on calpains in their life cycles. Whether calpain activity is involved in infections of the other members of picornavirus family, would be relevant to investigate.

The data presented here shows the critical role of calpain activity in EV1 life cycle, although revealing the exact target of calpains requires further investigations. The missing definable cleavage specifity of calpains complicates the assessment of their input into a given molecule´s processing. One of the complexities in studying calpains is also that the isoforms can substitute for each other. Novel approaches are required to solve how calpains find their targets in the right place at the right time.

To summarize, the data reported in this thesis is represented as a model for EV1 life cycle in Figure 11.

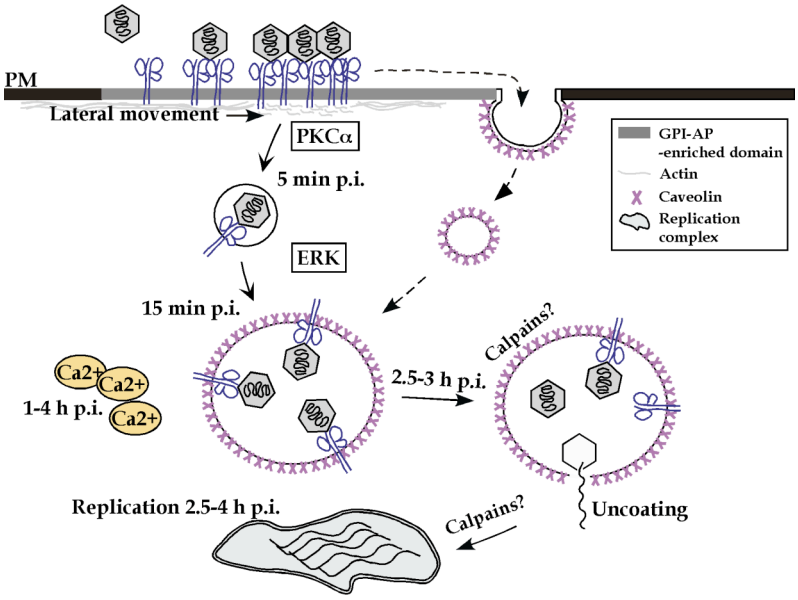


FIGURE 11 A model for EV1 life cycle. Ligand-free integrin localizes to GPI-AP enriched plasma membrane domains. Binding of EV1 to α2β1 integrin induces clustering and lateral movement of integrins along the cortical actin filaments. Internalization of EV1 and α2β1 integrin in small vesicles is regulated by PKCα. The vesicles fuse with caveosomes 15-30 min p.i. Transient activation of ERK occurs 15 min p.i. EV1 RNA is released from caveosomes to the cytoplasm for replication, which occurs on the membraneous vesicles, called replication complexes. The activity of calpains is probably required for the formation of replication complexes. Alternatively, calpains may have a role in the degradation of caveosomes. EV1 infection leads to an increase in the cytosolic Ca^{2+} level, possibly contributing to the calpain activity. In addition to the primary noncaveolar route, EV1 may use the caveolar-mediated entry route (indicated with dashed arrows).

7 CONCLUSIONS

The main conclusions of this thesis are:

1. EV1 together with its receptor α2β1 integrin internalize into caveosomes primarily through caveolae-independent endocytosis.

2. EV1, as a multivalent ligand, can trigger clustering of receptors and internalization processes in a similar way as antibodies. Internalization is dependent on PKCα activity and signaling events.

3. Cellular calpains are essential for the replication of EV1.

Acknowledgements

This work was carried out at the Department of Biological and Environmental Science/Nanoscience center, University of Jyväskylä. I thank my reviewers Professor Elina Ikonen and Adjunct Professor Eeva-Liisa Eskelinen for valuable advices and criticism to improve the final version of this thesis. Emily Knott is acknowledged for revising the language.

I wish to express my sincere thanks to my supervisor, Professor Jyrki Heino for providing me the opportunity to work in a talented team of Incredible Integrins. His wise and fair way to guide his students and the many gatherings he has offered his students outside the lab, have been worth a lot.

I am extremely grateful to my second supervisor, Dr. Varpu Marjomäki. It has been a pleasure to work with Varpu, whose expertise on science and endless kindness, patience, energy and support are admirable.

I am also deeply grateful to Käspi, who I had pleasure to share office room with before he moved to Turku. The many discussions and jokes we have had and his irreplaceable help with computers are memorable.

I wish to thank our Allu and the other lab technicians Pirjo, Ine and Eila for their kind help and contribution to the smoothness of experimental work. Raija and Paavo are acknowledged for their technical assistance in electron microscopy.

The co-authors and collaborators are warmly acknowledged. Especially Professor Timo Hyypiä´s expertise on the virology field has been valuable.

My special thanks go to the current and former members of the Incredible Integrins team: Mira, Hilkka, Liisa, Sanna L., Johanna, Camilla, Pasi, Pete, Dan, Wendy, Arsi, Pekka K., Tuomas, Pekka R., Abbi, Maria and Matti. Additionally, I want to thank the team Mighty Metalloproteinases for the amusing seminar trips. I thank my coworkers and colleagues for the pleasant atmosphere to work in and for the help whenever I needed it. Particularly, the current members of Varpu´s group are thanked: Elina, Mikko, Nina and Lassi. Thank you Sanna S. for helping me with the styling of this thesis. I also want to thank the former member Heli. I am thankful to Laura for being my dear friend and for our unforgettable mushroom trips and other funny occations. Thank you Sanna K., Anna and Kirsi for the great company and friendship, especially during the conference journeys. I wish to thank all the MOTU people for the co-operation.

I want to thank my friends outside the scientific world. Especially Sike, thank you for being the best friend in the world. My "backyard" friends are given thanks for balancing my life with all the enjoyable socials.

I am grateful to my family for always encouraging me and also for the financial support. Pauliina deserves special thanks for being my "little sister".

The study was supported by the National Graduate School in Nanoscience and by grants from the Academy of Finland, the Sigrid Jusélius Foundation, the Finnish Cancer Association, the Emil Aaltonen Foundation, the Ida Montin Foundation and the Foundation for Virological Studies in Finland.

YHTEENVETO (Résumé in Finnish)

Echovirus 1:n integriinivälitteinen sisäänmeno soluun

Echovirus 1 (EV1) on pieni, vaipaton RNA-virus ja osa pikornavirusperhettä, jonka muita tunnettuja jäseniä ovat mm. poliovirus sekä suu- ja sorkkatautivirus. EV1 on ihmisen virus, joka voi aiheuttaa aivokalvontulehdusta ja halvauksia mutta myös lievempiä hengitystie- ja suolistotulehduksia. Viruksen lisääntymiskierron vaiheet ovat viruksen tarttuminen solun pintaan, pääsy solun sisään ja perimän vapautuminen monistumista varten. Päästäkseen solun sisään EV1 tarttuu solun pinnan vastaanottajamolekyyliinsä, α2β1-integriini -reseptoriin. Integriinit ovat reseptoriperhe, jonka jäsenet pääasiallisesti välittävät solujen kiinnittymistä. Integriinit koostuvat α ja β -alayksiköistä muodostaen solukalvon läpäisevän parin, jonka solun ulkopuolinen osa kiinnittyy soluväliaineeseen ja solunsisäinen häntäosa aktiinitukirankaan. EV1:n sitoutumispaikka on α-alayksikön erillisessä I-domeenissa, johon myös α2β1-integriinin luonnollinen ligandi, kollageeni, sitoutuu. Ligandin sitoutuminen integriiniin johtaa reseptorien yhteenkertymiseen, mikä puolestaan on välttämätöntä monenlaisten viestinvälitykseen kuuluvien proteiinien liittymiseksi integriinin häntäosiin jatkamaan signaalinsiirtoa. Virus, joka käyttää tiettyä reseptoria, toimii erinomaisena työkaluna myös reseptorin luonnollisen toiminnan selvittämisessä. Molekyylitason mekanismeja, joilla pikornavirukset aiheuttavat sairauksia, ymmärretään huonosti. Jotta infektioita voidaan diagnosoida, hoitaa ja ennaltaehkäistä rokotteilla, on välttämätöntä selvittää viruksen reitti ja mukana toimivat proteiinit. On tärkeää saada tietoa myös integriinien toiminnasta, sillä häiriöt niissä voivat johtaa vakaviin sairauksiin, kuten syöpään, veritulppiin ja tulehduksiin.

Solun sisäänmenoonsa eri pikornavirukset käyttävät erilaisia tapoja. Elottomana mutta muuntuvana partikkelina virus hyödyntää täysin solun normaaleja toimintoja, mukaanlukien endosytoosi- ja proteiininvalmistuskoneistoja. Usein virukset pääsevät soluun klatriinivuoratuissa rakkuloissa mutta EV1 ei käyttänyt tätä reittiä. EV1:n pitkäkestoinen vuorovaikutus α2β1-integriinin kanssa alkoi, kun reseptori seurasi virusta solun tumanviereisiin rakkularakenteisiin, kaveosomeihin. Kaveosomit ovat kaveoliini-1 –positiivisia, neutraaleja soluelimiä. Reseptoriin kiinnittyminen ei aikaansaanut EV1:ssa RNA-perimän vapautumiseen tarvittavaa konformaation muutosta, vaan perimä vapautui vasta viruksen päästyä solun sisään.

Tärkeitä EV1-infektion kannalta ovat solukalvon alueet, jotka sisältävät kolesterolia, rasvoja ja viestinsiirtoon osallistuvia proteiineja. Ligandittoman α2β1-integriinin nähtiin sijaitsevan näillä solukalvon alueilla. Kun usean integriinin yhteenkertyminen aiheutettiin vasta-aineilla, reseptorit liikkuivat kalvonalaisia aktiinisäikeitä pitkin, kunnes solahtivat solun sisään. Prosessi oli riippuvainen proteiini kinaasi Cα:n aktiivisuudesta. EV1:n tiedetään pystyvän sitomaan yhtäaikaa 60 integriinireseptoriparia ja samoin kuin vasta-aineet, aikaansaamaan reseptorien yhteenkertymisen. Tämä on riittävää integriinien saamiseksi solun sisään, jonne

virus pääsee mukana.

Aluksi oletuksena oli, että EV1 pääsee soluun kaveolien, solukalvon kuopakkeiden kautta mutta yhteisinfektio kaveolireittiä käyttävän simian virus 40:n (SV40) kanssa osoitti, että EV1:n sisäänmeno oli paljon nopeampi ja poikkesi viestinsiirron osaltakin SV40:n reitistä. Näinollen EV1 aloittaa soluun menonsa eri reittiä kuin SV40 mutta virusten nähtiin kohtaavan myöhemmin kaveosomeissa, EV:ta sisältävien kuljetusrakkuloiden todennäköisesti fuusioituessa niihin.

Pikornavirukset monistuvat solulimassa sitoutuneena muokkaamiinsa kalvorakenteisiin. Radioaktiivisella EV1:lla tehdyt sedimentaatioanalyysit osoittivat, että tyhjiä viruspartikkeleita, joista RNA-perimä on vapautunut, alkoi ilmestyä 30 min-2 h infektion jälkeen. Kaveosomeista EV1:n proteiinikuoren osat eivät näyttäneet siirtyvän eteenpäin muihin soluelimiin eli perimän täytyi vapautua sieltä suoraan solulimaan. Saattaa olla että α2β1-integriinin I-domeenin vakauttava vaikutus esti siihen saakka rakennetta väljentävät muutokset viruksen proteiinikuoressa. Pikornaviruksen RNA toimii suoraan lähetti-RNA:na, jonka koodaamasta pitkästä proteiinista pilkotaan viruksen rakennusaineet; viruskuoriproteiinit ja monistumisessa tarvittavat proteiinit.

EV1-infektion kannalta kriittisiä olivat solun kalsiumista riippuvaiset kysteiiniproteaasit, kalpaiini-1 ja kalpaiini-2. Ne säätelevät monia solun toimintoja pilkkomalla tai muotoilemalla kohdeproteiinejaan, joita ovat mm. aktiiniin liittyvät solutukirangan proteiinit, erilaiset kinaasit ja GTPaasit. Kalpaiinit pilkkovat myös β-integriinien solunsisäisiä häntiä ja tämän vuoksi niiden mahdollista osallisuutta EV1-infektioon alettiin tutkia. Kalpaiinien estäminen aiheutti infektion estymisen paitsi kemiallisilla inhibiittoreilla, myös erityisillä siRNA:lla. Myös muut testatut pikornavirukset, HPEV1 ja CVB3 olivat infektioissaan riippuvaisia kalpaiiniaktiivisuudesta. Kalpaiinit toimivat vasta myöhemmissä infektion vaiheissa, sillä niillä ei ollut vaikutusta viruksen tai integriinin pääsyyn tai sijaintiin solussa. Se, että EV1:n RNA mikroinjektoituna kalpaiiniestäjän läsnäollessa ei johtanut infektioon osoitti, että kalpaiinien rooli on vasta perimän vapautumisen jälkeen. Kalpaiinien täsmällinen vaikutuskohde EV1-infektiossa jää tutkittavaksi. Kalpaiinit voivat mahdollisesti muokata ei-rakenneproteiinien aktiivisuuksia ja sitä kautta vaikuttaa monistumisessa tarvittavien kalvorakennelmien rakentamiseen.

REFERENCES

Abraham, G. & Colonno, R.J. 1984. Many rhinovirus serotypes share the same cellular receptor. J. Virol. 51, 340-345.

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D. & Brown, F. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution. Nature 337, 709-716.

Al-Sunaidi, M., Williams, C.H., Hughes, P.J., Schnurr, D.P. & Stanway, G. 2007. Analysis of a new human parechovirus allows the definition of parechovirus types and the identification of RNA structural domains. J. Virol. 81, 1013-1021.

Andino, R., Rieckhof, G.E. & Baltimore, D. 1990. A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA. Cell 63, 369-380.

Andreoletti, L., Hober, D., Hober-Vandenberghe, C., Belaich, S., Vantyghem, M.C., Lefebvre, J. & Wattre, P. 1997. Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. J. Med. Virol. 52, 121-127.

Anton, I.M., Saville, S.P., Byrne, M.J., Curcio, C., Ramesh, N., Hartwig, J.H. & Geha, R.S. 2003. WIP participates in actin reorganization and ruffle formation induced by PDGF. J. Cell. Sci. 116, 2443-2451.

Araki, N., Egami, Y., Watanabe, Y. & Hatae, T. 2007. Phosphoinositide metabolism during membrane ruffling and macropinosome formation in EGF-stimulated A431 cells. Exp. Cell. Res. 313, 1496-1507.

Araki, N., Johnson, M.T. & Swanson, J.A. 1996. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J. Cell Biol. 135, 1249-1260.

Arthur, J.S. & Crawford, C. 1996. Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. Biochim. Biophys. Acta 1293, 201-206.

Arthur, J.S., Elce, J.S., Hegadorn, C., Williams, K. & Greer, P.A. 2000. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol. Cell. Biol. 20, 4474-4481.

Ashby, M.C. & Tepikin, A.V. 2001. ER calcium and the functions of intracellular organelles. Semin. Cell. Dev. Biol. 12, 11-17.

Auvinen, P., Mäkelä, M.J., Roivainen, M., Kallajoki, M., Vainionpää, R. & Hyypiä, T. 1993. Mapping of antigenic sites of coxsackievirus B3 by synthetic peptides. APMIS 101, 517-528.

Averna, M., Stifanese, R., De Tullio, R., Defranchi, E., Salamino, F., Melloni, E. & Pontremoli, S. 2006. Interaction between catalytically inactive calpain and calpastatin. Evidence for its occurrence in stimulated cells. FEBS J. 273, 1660-1668.

Back, S.H., Kim, Y.K., Kim, W.J., Cho, S., Oh, H.R., Kim, J.E. & Jang, S.K. 2002. Translation of polioviral mRNA is inhibited by cleavage of polypyrimidine tract-binding proteins executed by polioviral 3C(pro). J. Virol. 76, 2529-2542.

Baki, A., Tompa, P., Alexa, A., Molnar, O. & Friedrich, P. 1996. Autolysis parallels

activation of mu-calpain. Biochem. J. 318, 897-901.
Banerjee, R., Echeverri, A. & Dasgupta, A. 1997. Poliovirus-encoded 2C polypeptide specifically binds to the 3'-terminal sequences of viral negative-strand RNA. J. Virol. 71, 9570-9578.
Banting, G., Maile, R. & Roquemore, E.P. 1998. The steady state distribution of humTGN46 is not significantly altered in cells defective in clathrin-mediated endocytosis. J. Cell. Sci. 111, 3451-3458.
Barnstable, C.J., Jones, E.A. & Crumpton, M.J. 1978. Isolation, structure and genetics of HLA-A, -B, -C and -DRw (Ia) antigens. Br. Med. Bull. 34, 241-246.
Barton, D.J., O'Donnell, B.J. & Flanegan, J.B. 2001. 5' cloverleaf in poliovirus RNA is a cis-acting replication element required for negative-strand synthesis. EMBO J. 20, 1439-1448.
Beckerle, M.C., Burridge, K., DeMartino, G.N. & Croall, D.E. 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. Cell 51, 569-577.
Beglova, N., Blacklow, S.C., Takagi, J. & Springer, T.A. 2002. Cysteine-rich module structure reveals a fulcrum for integrin rearrangement upon activation. Nat. Struct. Biol. 9, 282-287.
Bellve, K.D., Leonard, D., Standley, C., Lifshitz, L.M., Tuft, R.A., Hayakawa, A., Corvera, S. & Fogarty, K.E. 2006. Plasma membrane domains specialized for clathrin-mediated endocytosis in primary cells. J. Biol. Chem. 281, 16139-16146.
Belnap, D.M., McDermott, B.M., Jr., Filman, D.J., Cheng, N., Trus, B.L., Zuccola, H.J., Racaniello, V.R., Hogle, J.M. & Steven, A.C. 2000. Three-dimensional structure of poliovirus receptor bound to poliovirus. Proc. Natl. Acad. Sci. USA 97, 73-78.
Belov, G.A., Evstafieva, A.G., Rubtsov, Y.P., Mikitas, O.V., Vartapetian, A.B. & Agol, V.I. 2000. Early alteration of nucleocytoplasmic traffic induced by some RNA viruses. Virology 275, 244-248.
Belsham, G.J. & Sonenberg, N. 2000. Picornavirus RNA translation: roles for cellular proteins. Trends Microbiol. 8, 330-335.
Benmerah, A., Bayrou, M., Cerf-Bensussan, N. & Dautry-Varsat, A. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J. Cell. Sci. 112, 1303-1311.
Benmerah, A. & Lamaze, C. 2007. Clathrin-Coated Pits: Vive La Difference? Traffic 8, 970-982.
Bergelson, J.M., Chan, B.M., Finberg, R.W. & Hemler, M.E. 1993a. The integrin VLA-2 binds echovirus 1 and extracellular matrix ligands by different mechanisms. J. Clin. Invest. 92, 232-239.
Bergelson, J.M., Chan, M., Solomon, K.R., St John, N.F., Lin, H. & Finberg, R.W. 1994. Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. Proc. Natl. Acad. Sci. USA 91, 6245-6248.
Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L. & Finberg, R.W. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science

275, 1320-1323.

Bergelson, J.M., Mohanty, J.G., Crowell, R.L., St John, N.F., Lublin, D.M. & Finberg, R.W. 1995. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). J. Virol. 69, 1903-1906.

Bergelson, J.M., Shepley, M.P., Chan, B.M., Hemler, M.E. & Finberg, R.W. 1992. Identification of the integrin VLA-2 as a receptor for echovirus 1. Science 275, 1718-1720.

Bergelson, J.M., St John, N., Kawaguchi, S., Chan, M., Stubdal, H., Modlin, J. & Finberg, R.W. 1993b. Infection by echoviruses 1 and 8 depends on the alpha 2 subunit of human VLA-2. J. Virol. 67, 6847-6852.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P.W. & Baxt, B. 1995. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. 69, 2664-2666.

Bialkowska, K., Kulkarni, S., Du, X., Goll, D.E., Saido, T.C. & Fox, J.E. 2000. Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. J.Cell Biol. 151, 685-696.

Bolten, R., Egger, D., Gosert, R., Schaub, G., Landmann, L. & Bienz, K. 1998. Intracellular localization of poliovirus plus- and minus-strand RNA visualized by strand-specific fluorescent In situ hybridization. J. Virol. 72, 8578-8585.

Bonazzi, M., Spano, S., Turacchio, G., Cericola, C., Valente, C., Colanzi, A., Kweon, H.S., Hsu, V.W., Polishchuck, E.V., Polishchuck, R.S., Sallese, M., Pulvirenti, T., Corda, D. & Luini, A. 2005. CtBP3/BARS drives membrane fission in dynamin-independent transport pathways. Nat. Cell. Biol. 7, 570-580.

Borman, A.M., Kirchweger, R., Ziegler, E., Rhoads, R.E., Skern, T. & Kean, K.M. 1997. elF4G and its proteolytic cleavage products: effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. RNA 3, 186-196.

Botos, E., Klumperman, J., Oorschot, V., Igyarto, B., Magyar, A., Olah, M. & Kiss, A.L. 2007. Caveolin-1 is transported to multivesicular bodies after albumin-induced endocytosis of caveolae in HepG2 cells. J.Cell. Mol. Med. (Epub ahead of print)

Brandenburg, B., Lee, L.Y., Lakadamyali, M., Rust, M.J., Zhuang, X. & Hogle, J.M. 2007. Imaging Poliovirus Entry in Live Cells. PLoS Biol. 5, e183.

Bretscher, M.S. 1992. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J. 11, 405-410.

Bretscher, M.S. 1996. Moving membrane up to the front of migrating cells. Cell 85, 465-467.

Brunner, J.E., Nguyen, J.H., Roehl, H.H., Ho, T.V., Swiderek, K.M. & Semler, B.L. 2005. Functional interaction of heterogeneous nuclear ribonucleoprotein C with poliovirus RNA synthesis initiation complexes. J. Virol. 79, 3254-3266.

Bryant, D.M., Kerr, M.C., Hammond, L.A., Joseph, S.R., Mostov, K.E., Teasdale, R.D. & Stow, J.L. 2007. EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. J. Cell. Sci. 120, 1818-1828.

Cairo, C.W., Mirchev, R. & Golan, D.E. 2006. Cytoskeletal regulation couples LFA-1 conformational changes to receptor lateral mobility and clustering. Immunity 25, 297-308.

Calderwood, D.A. 2004. Integrin activation. J. Cell. Sci. 117, 657-666.

Cao, H., Garcia, F. & McNiven, M.A. 1998. Differential distribution of dynamin isoforms in mammalian cells. Mol. Biol. Cell 9, 2595-2609.

Carragher, N.O., Levkau, B., Ross, R. & Raines, E.W. 1999. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J. Cell Biol. 147, 619-630.

Carter, J. & Saunders, V. 2007. Virology, principles and applications. John Wiley & Sons, Ltd, p. 161.

Chen, Z., Knutson, E., Kurosky, A. & Albrecht, T. 2001. Degradation of p21cip1 in cells productively infected with human cytomegalovirus. J. Virol. 75, 3613-3625.

Chow, M., Newman, J.F., Filman, D., Hogle, J.M., Rowlands, D.J. & Brown, F. 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature 327, 482-486.

Chung, S.K., Kim, J.Y., Kim, I.B., Park, S.I., Paek, K.H. & Nam, J.H. 2005. Internalization and trafficking mechanisms of coxsackievirus B3 in HeLa cells. Virology 333, 31-40.

Claas, C., Stipp, C.S. & Hemler, M.E. 2001. Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts. J. Biol. Chem. 276, 7974-7984.

Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S.Q., Trowbridge, I.S. & Tainer, J.A. 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. Cell 63, 1061-1072.

Colonno, R.J., Condra, J.H., Mizutani, S., Callahan, P.L., Davies, M.E. & Murcko, M.A. 1988. Evidence for the direct involvement of the rhinovirus canyon in receptor binding. Proc. Natl. Acad. Sci. USA 85, 5449-5453.

Connors, W.L., Jokinen, J., White, D.J., Puranen, J.S., Kankaanpaa, P., Upla, P., Tulla, M., Johnson, M.S. & Heino, J. 2007. Two synergistic activation mechanisms of alpha2beta1 integrin-mediated collagen binding. J. Biol. Chem. 282, 14675-14683.

Coyne, C.B., Kim, K.S. & Bergelson, J.M. 2007. Poliovirus entry into human brain microvascular cells requires receptor-induced activation of SHP-2. EMBO J. 26, 4016-4028.

Curry, S., Chow, M. & Hogle, J.M. 1996. The poliovirus 135S particle is infectious. J. Virol. 70, 7125-7131.

D'Souza-Schorey, C., van Donselaar, E., Hsu, V.W., Yang, C., Stahl, P.D. & Peters, P.J. 1998. ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. J. Cell Biol. 140, 603-616.

Damm, E.M., Pelkmans, L., Kartenbeck, J., Mezzacasa, A., Kurzchalia, T. & Helenius, A. 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J. Cell Biol. 168, 477-488.

Danthi, P., Tosteson, M., Li, Q.H. & Chow, M. 2003. Genome delivery and ion

channel properties are altered in VP4 mutants of poliovirus. J. Virol. 77, 5266-5274.

Debiasi, R.L., Squier, M.K., Pike, B., Wynes, M., Dermody, T.S., Cohen, J.J. & Tyler, K.L. 1999. Reovirus-induced apoptosis is preceded by increased cellular calpain activity and is blocked by calpain inhibitors. J. Virol. 73, 695-701.

Decker, L. & ffrench-Constant, C. 2004. Lipid rafts and integrin activation regulate oligodendrocyte survival. J. Neurosci. 24, 3816-3825.

del Pozo, M.A., Alderson, N.B., Kiosses, W.B., Chiang, H.H., Anderson, R.G. & Schwartz, M.A. 2004. Integrins regulate Rac targeting by internalization of membrane domains. Science 303, 839-842.

Dharmawardhane, S., Schurmann, A., Sells, M.A., Chernoff, J., Schmid, S.L. & Bokoch, G.M. 2000. Regulation of macropinocytosis by p21-activated kinase-1. Mol. Biol. Cell 11, 3341-3352.

Dinter, A. & Berger, E.G. 1998. Golgi-disturbing agents. Histochem. Cell Biol. 109, 571-590.

Dorner, A.J., Semler, B.L., Jackson, R.J., Hanecak, R., Duprey, E. & Wimmer, E. 1984. In vitro translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol. 50, 507-514.

Elce, J.S., Hegadorn, C. & Arthur, J.S. 1997. Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. J. Biol. Chem. 272, 11268-11275.

Elices, M.J. & Hemler, M.E. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Natl. Acad. Sci. USA 86, 9906-9910.

Emsley, J., Knight, C.G., Farndale, R.W., Barnes, M.J. & Liddington, R.C. 2000. Structural basis of collagen recognition by integrin alpha2beta1. Cell 101, 47-56.

Etchison, D. & Fout, S. 1985. Human rhinovirus 14 infection of HeLa cells results in the proteolytic cleavage of the p220 cap-binding complex subunit and inactivates globin mRNA translation in vitro. J. Virol. 54, 634-638.

Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N. & Hershey, J.W. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. 257, 14806-14810.

Ezratty, E.J., Partridge, M.A. & Gundersen, G.G. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat. Cell Biol. 7, 581-590.

Feig, L.A. & Cooper, G.M. 1988. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol. Cell. Biol. 8, 3235-3243.

Feire, A.L., Koss, H. & Compton, T. 2004. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc. Natl. Acad. Sci. USA 101, 15470-15475.

Filman, D.J., Syed, R., Chow, M., Macadam, A.J., Minor, P.D. & Hogle, J.M. 1989. Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. EMBO J. 8, 1567-1579.

Filman, D.J., Wien, M.W., Cunningham, J.A., Bergelson, J.M. & Hogle, J.M. 1998.

Structure determination of echovirus 1. Acta Crystallogr. D. Biol. Crystallogr. 54, 1261-1272.

Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P.E., Parton, R.G. & van der Goot, F.G. 2002. Differential sorting and fate of endocytosed GPI-anchored proteins. EMBO J. 21, 3989-4000.

Ford, M.G., Pearse, B.M., Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R. & McMahon, H.T. 2001. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 291, 1051-1055.

Fox, J.E., Reynolds, C.C. & Phillips, D.R. 1983. Calcium-dependent proteolysis occurs during platelet aggregation. J. Biol. Chem. 258, 9973-9981.

Fox, J.E., Taylor, R.G., Taffarel, M., Boyles, J.K. & Goll, D.E. 1993. Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb-IIIa. J. Cell Biol. 120, 1501-1507.

Franco, S., Perrin, B. & Huttenlocher, A. 2004. Isoform specific function of calpain 2 in regulating membrane protrusion. Exp. Cell. Res. 299, 179-187.

Franco, S.J. & Huttenlocher, A. 2005. Regulating cell migration: calpains make the cut. J. Cell. Sci. 118, 3829-3838.

Fricks, C.E. & Hogle, J.M. 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. 64, 1934-1945.

Gagescu, R., Demaurex, N., Parton, R.G., Hunziker, W., Huber, L.A. & Gruenberg, J. 2000. The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. Mol. Biol. Cell 11, 2775-2791.

Galbraith, C.G., Yamada, K.M. & Galbraith, J.A. 2007. Polymerizing actin fibers position integrins primed to probe for adhesion sites. Science 315, 992-995.

Gamarnik, A.V. & Andino, R. 1998. Switch from translation to RNA replication in a positive-stranded RNA virus. Genes Dev. 12, 2293-2304.

Gaus, K., Le Lay, S., Balasubramanian, N. & Schwartz, M.A. 2006. Integrin-mediated adhesion regulates membrane order. J. Cell Biol. 174, 725-734.

Gerber, K., Wimmer, E. & Paul, A.V. 2001. Biochemical and genetic studies of the initiation of human rhinovirus 2 RNA replication: identification of a cis-replicating element in the coding sequence of 2A(pro). J. Virol. 75, 10979-10990.

Glading, A., Bodnar, R.J., Reynolds, I.J., Shiraha, H., Satish, L., Potter, D.A., Blair, H.C. & Wells, A. 2004. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol. Cell. Biol. 24, 2499-2512.

Goll, D.E., Thompson, V.F., Li, H., Wei, W. & Cong, J. 2003. The calpain system. Physiol. Rev. 83, 731-801.

Goodfellow, I., Chaudhry, Y., Richardson, A., Meredith, J., Almond, J.W., Barclay, W. & Evans, D.J. 2000. Identification of a cis-acting replication element within the poliovirus coding region. J. Virol. 74, 4590-4600.

Graham, K.L., Halasz, P., Tan, Y., Hewish, M.J., Takada, Y., Mackow, E.R., Robinson, M.K. & Coulson, B.S. 2003. Integrin-using rotaviruses bind

alpha2beta1 integrin alpha2 I domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during cell entry. J. Virol. 77, 9969-9978.

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E. & McClelland, A. 1989. The major human rhinovirus receptor is ICAM-1. Cell 56, 839-847.

Grimmer, S., van Deurs, B. & Sandvig, K. 2002. Membrane ruffling and macropinocytosis in A431 cells require cholesterol. J. Cell. Sci. 115, 2953-2962.

Gruenberg, J. 2001. The endocytic pathway: a mosaic of domains. Nature Rev. Mol. Cell Biol. 2, 721-730.

Grunert, H.P., Wolf, K.U., Langner, K.D., Sawitzky, D., Habermehl, K.O. & Zeichhardt, H. 1997. Internalization of human rhinovirus 14 into HeLa and ICAM-1-transfected BHK cells. Med. Microbiol. Immunol. 186, 1-9.

Gu, F. & Gruenberg, J. 1999. Biogenesis of transport intermediates in the endocytic pathway. FEBS Lett 452, 61-66.

Guroff, G. 1964. A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain. J. Biol. Chem. 239, 149-155.

Gustin, K.E. & Sarnow, P. 2001. Effects of poliovirus infection on nucleo-cytoplasmic trafficking and nuclear pore complex composition. EMBO J. 20, 240-249.

Guttmann, R.P., Elce, J.S., Bell, P.D., Isbell, J.C. & Johnson, G.V. 1997. Oxidation inhibits substrate proteolysis by calpain I but not autolysis. J. Biol. Chem. 272, 2005-2012.

Harris, K.S., Xiang, W., Alexander, L., Lane, W.S., Paul, A.V. & Wimmer, E. 1994. Interaction of poliovirus polypeptide 3CDpro with the 5' and 3' termini of the poliovirus genome. Identification of viral and cellular cofactors needed for efficient binding. J. Biol. Chem. 269, 27004-27014.

Hellen, C.U. & Wimmer, E. 1995. Translation of encephalomyocarditis virus RNA by internal ribosomal entry. Curr. Top. Microbiol. Immunol. 203, 31-63.

Hendry, E., Hatanaka, H., Fry, E., Smyth, M., Tate, J., Stanway, G., Säntti, J., Maaronen, M., Hyypiä, T. & Stuart, D. 1999. The crystal structure of coxsackievirus A9: new insights into the uncoating mechanisms of enteroviruses. Structure 7, 1527-1538.

Henley, J.R., Krueger, E.W., Oswald, B.J. & McNiven, M.A. 1998. Dynamin-mediated internalization of caveolae. J. Cell Biol. 141, 85-99.

Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E. & Blaas, D. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. Proc. Natl. Acad. Sci. USA 91, 1839-1842.

Hogle, J.M. 2002. Poliovirus cell entry: common structural themes in viral cell entry pathways. Annu. Rev. Microbiol. 56, 677-702.

Hogle, J.M., Chow, M. & Filman, D.J. 1985. Three-dimensional structure of poliovirus at 2.9 A resolution. Science (New York, N.Y 229, 1358-1365.

Holmström, T.H., Tran, S.E., Johnson, V.L., Ahn, N.G., Chow, S.C. & Eriksson, J.E. 1999. Inhibition of mitogen-activated kinase signaling sensitizes HeLa cells to Fas receptor-mediated apoptosis. Mol. Cell. Biol. 19, 5991-6002.

Hood, J.L., Brooks, W.H. & Roszman, T.L. 2004. Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus. J. Biol. Chem. 279, 43126-43135.

Hood, J.L., Brooks, W.H. & Roszman, T.L. 2006. Subcellular mobility of the calpain/calpastatin network: an organelle transient. Bioessays 28, 850-859.

Hood, J.L., Logan, B.B., Sinai, A.P., Brooks, W.H. & Roszman, T.L. 2003. Association of the calpain/calpastatin network with subcellular organelles. Biochem. Biophys. Res. Commun. 310, 1200-1212.

Hoover-Litty, H. & Greve, J.M. 1993. Formation of rhinovirus-soluble ICAM-1 complexes and conformational changes in the virion. J. Virol. 67, 390-397.

Hosfield, C.M., Elce, J.S., Davies, P.L. & Jia, Z. 1999. Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18, 6880-6889.

Hotchin, N.A. & Hall, A. 1995. The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. J. Cell Biol. 131, 1857-1865.

Huber, S.A. 1994. VCAM-1 is a receptor for encephalomyocarditis virus on murine vascular endothelial cells. J. Virol. 68, 3453-3458.

Huovila, A.P., Eder, A.M. & Fuller, S.D. 1992. Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J. Cell Biol. 118, 1305-1320.

Huttenlocher, A., Palecek, S.P., Lu, Q., Zhang, W., Mellgren, R.L., Lauffenburger, D.A., Ginsberg, M.H. & Horwitz, A.F. 1997. Regulation of cell migration by the calcium-dependent protease calpain. J. Biol. Chem. 272, 32719-32722.

Huttunen, P., Heino, J. & Hyypiä, T. 1997. Echovirus 1 replication, not only virus binding to its receptor, VLA-2, is required for the induction of cellular immediate-early genes. J. Virol. 71, 4176-4180.

Huttunen, P., Hyypiä, T., Vihinen, P., Nissinen, L. & Heino, J. 1998. Echovirus 1 infection induces both stress- and growth-activated mitogen-activated protein kinase pathways and regulates the transcription of cellular immediate-early genes. Virology 250, 85-93.

Hynes, R.O. 2002. Integrins: bidirectional, allosteric signaling machines. Cell 110, 673-687.

Hyypiä, T., Horsnell, C., Maaronen, M., Khan, M., Kalkkinen, N., Auvinen, P., Kinnunen, L. & Stanway, G. 1992. A distinct picornavirus group identified by sequence analysis. Proc. Natl. Acad. Sci. USA 89, 8847-8851.

Irurzun, A., Arroyo, J., Alvarez, A. & Carrasco, L. 1995. Enhanced intracellular calcium concentration during poliovirus infection. J. Virol. 69, 5142-5146.

Ito, M., Yamashita, T., Tsuzuki, H., Takeda, N. & Sakae, K. 2004. Isolation and identification of a novel human parechovirus. J. Gen. Virol. 85, 391-398.

Ivaska, J., Reunanen, H., Westermarck, J., Koivisto, L., Kähäri, V.M. & Heino, J. 1999. Integrin alpha2beta1 mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the alpha2 cytoplasmic tail. J. Cell Biol. 147, 401-416.

Ivaska, J., Whelan, R.D., Watson, R. & Parker, P.J. 2002. PKC epsilon controls the traffic of beta1 integrins in motile cells. EMBO J. 21, 3608-3619.

Jackson, T., Ellard, F.M., Ghazaleh, R.A., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W. & King, A.M. 1996. Efficient infection of

cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. J. Virol. 70, 5282-5287.

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W. & King, A.M. 2000. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. J. Virol. 74, 4949-4956.

Jackson, W.T., Giddings, T.H., Jr., Taylor, M.P., Mulinyawe, S., Rabinovitch, M., Kopito, R.R. & Kirkegaard, K. 2005. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol. 3, e156.

Jaken, S., Leach, K. & Klauck, T. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. J. Cell Biol. 109, 697-704.

Jang, S.K., Krausslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C. & Wimmer, E. 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. 62, 2636-2643.

Joki-Korpela, P. & Hyypiä, T. 1998. Diagnosis and epidemiology of echovirus 22 infections. Clin. Infect. Dis. 27, 129-136.

Joki-Korpela, P., Marjomäki, V., Krogerus, C., Heino, J. & Hyypiä, T. 2001. Entry of human parechovirus 1. J. Virol. 75, 1958-1967.

Kalamvoki, M. & Mavromara, P. 2004. Calcium-dependent calpain proteases are implicated in processing of the hepatitis C virus NS5A protein. J. Virol. 78, 11865-11878.

Katpally, U. & Smith, T.J. 2007. Pocket factors unlikely play a major role in the life cycle of human rhinovirus. J. Virol. 81, 6307-6315.

Kerr, M.C., Lindsay, M.R., Luetterforst, R., Hamilton, N., Simpson, F., Parton, R.G., Gleeson, P.A. & Teasdale, R.D. 2006. Visualisation of macropinosome maturation by the recruitment of sorting nexins. J. Cell. Sci. 119, 3967-3980.

Khorchid, A. & Ikura, M. 2002. How calpain is activated by calcium. Nat. Struct. Biol. 9, 239-241.

Kifor, O., Kifor, I., Moore, F.D., Jr., Butters, R.R., Jr. & Brown, E.M. 2003. m-Calpain colocalizes with the calcium-sensing receptor (CaR) in caveolae in parathyroid cells and participates in degradation of the CaR. J. Biol. Chem. 278, 31167-31176.

Kim, M., Carman, C.V. & Springer, T.A. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science 301, 1720-1725.

Kim, M., Carman, C.V., Yang, W., Salas, A. & Springer, T.A. 2004. The primacy of affinity over clustering in regulation of adhesiveness of the integrin {alpha}L{beta}2. J. Cell Biol. 167, 1241-1253.

Kim, S.S., Smith, T.J., Chapman, M.S., Rossmann, M.C., Pevear, D.C., Dutko, F.J., Felock, P.J., Diana, G.D. & McKinlay, M.A. 1989. Crystal structure of human rhinovirus serotype 1A (HRV1A). J. Mol. Biol. 210, 91-111.

King, S.L., Cunningham, J.A., Finberg, R.W. & Bergelson, J.M. 1995. Echovirus 1 interaction with the isolated VLA-2 I domain. J. Virol. 69, 3237-3239.

Kirkham, M., Fujita, A., Chadda, R., Nixon, S.J., Kurzchalia, T.V., Sharma, D.K., Pagano, R.E., Hancock, J.F., Mayor, S. & Parton, R.G. 2005. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J. Cell Biol. 168, 465-476.

Kirkham, M. & Parton, R.G. 2005. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. Biochim. Biophys. Acta 1746, 349-363.

Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T. & Nishizuka, Y. 1989. Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J. Biol. Chem. 264, 4088-4092.

Kolatkar, P.R., Bella, J., Olson, N.H., Bator, C.M., Baker, T.S. & Rossmann, M.G. 1999. Structural studies of two rhinovirus serotypes complexed with fragments of their cellular receptor. EMBO J. 18, 6249-6259.

Kronenberger, P., Schober, D., Prchla, E., Ofori-Anyinam, O., Vrijsen, R., Rombaut, B., Blaas, D., Fuchs, R. & Boeye, A. 1998. Uptake of poliovirus into the endosomal system of HeLa cells. Arch. Virol. 143, 1417-1424.

Kulkarni, S., Goll, D.E. & Fox, J.E. 2002. Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. J. Biol. Chem. 277, 24435-24441.

Kulkarni, S., Saido, T.C., Suzuki, K. & Fox, J.E. 1999. Calpain mediates integrin-induced signaling at a point upstream of Rho family members. J. Biol. Chem. 274, 21265-21275.

Kumamoto, T., Kleese, W.C., Cong, J.Y., Goll, D.E., Pierce, P.R. & Allen, R.E. 1992. Localization of the Ca(2+)-dependent proteinases and their inhibitor in normal, fasted, and denervated rat skeletal muscle. Anatom. Rec. 232, 60-77.

Kwon, M.S., Park, C.S., Choi, K., Ahnn, J., Kim, J.I., Eom, S.H., Kaufman, S.J. & Song, W.K. 2000. Calreticulin couples calcium release and calcium influx in integrin-mediated calcium signaling. Mol. Biol. Cell 11, 1433-1443.

Lama, J., Paul, A.V., Harris, K.S. & Wimmer, E. 1994. Properties of purified recombinant poliovirus protein 3aB as substrate for viral proteinases and as co-factor for RNA polymerase 3Dpol. J. Biol. Chem. 269, 66-70.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C.G., Benmerah, A. & Dautry-Varsat, A. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol. Cell 7, 661-671.

Le, P.U. & Nabi, I.R. 2003. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. J. Cell. Sci. 116, 1059-1071.

Lee, J.O., Rieu, P., Arnaout, M.A. & Liddington, R. 1995. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). Cell 80, 631-638.

Leitinger, B. & Hogg, N. 2002. The involvement of lipid rafts in the regulation of integrin function. J. Cell. Sci. 115, 963-972.

Lentz, K.N., Smith, A.D., Geisler, S.C., Cox, S., Buontempo, P., Skelton, A., DeMartino, J., Rozhon, E., Schwartz, J., Girijavallabhan, V., O'Connell, J. & Arnold, E. 1997. Structure of poliovirus type 2 Lansing complexed with antiviral agent SCH48973: comparison of the structural and biological properties of three poliovirus serotypes. Structure 5, 961-978.

Liddington, R.C. & Ginsberg, M.H. 2002. Integrin activation takes shape. J. Cell Biol. 158, 833-839.

Lin, G.D., Chattopadhyay, D., Maki, M., Wang, K.K., Carson, M., Jin, L., Yuen,

P.W., Takano, E., Hatanaka, M., DeLucas, L.J. & Narayana, S.V. 1997. Crystal structure of calcium bound domain VI of calpain at 1.9 A resolution and its role in enzyme assembly, regulation, and inhibitor binding. Nat. Struct. Biol. 4, 539-547.

Liu, S., Calderwood, D.A. & Ginsberg, M.H. 2000. Integrin cytoplasmic domain-binding proteins. J. Cell. Sci. 113, 3563-3571.

Maldonado-Baez, L. & Wendland, B. 2006. Endocytic adaptors: recruiters, coordinators and regulators. Trends Cell. Biol. 16, 505-513.

Marjomäki, V., Pietiäinen, V., Matilainen, H., Upla, P., Ivaska, J., Nissinen, L., Reunanen, H., Huttunen, P., Hyypiä, T. & Heino, J. 2002. Internalization of echovirus 1 in caveolae. J. Virol. 76, 1856-1865.

Marjomäki, V.S., Huovila, A.P., Surkka, M.A., Jokinen, I. & Salminen, A. 1990. Lysosomal trafficking in rat cardiac myocytes. J. Histochem. Cytochem. 38, 1155-1164.

Marsh, M. & Helenius, A. 2006. Virus entry: open sesame. Cell 124, 729-740.

Mason, P.W., Bezborodova, S.V. & Henry, T.M. 2002. Identification and characterization of a cis-acting replication element (cre) adjacent to the internal ribosome entry site of foot-and-mouth disease virus. J. Virol. 76, 9686-9694.

McCarty, O.J., Zhao, Y., Andrew, N., Machesky, L.M., Staunton, D., Frampton, J. & Watson, S.P. 2004. Evaluation of the role of platelet integrins in fibronectin-dependent spreading and adhesion. J. Thromb. Haemost. 2, 1823-1833.

McKnight, K.L. & Lemon, S.M. 1998. The rhinovirus type 14 genome contains an internally located RNA structure that is required for viral replication. RNA 4, 1569-1584.

Meier, O., Boucke, K., Hammer, S.V., Keller, S., Stidwill, R.P., Hemmi, S. & Greber, U.F. 2002. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. J. Cell Biol. 158, 1119-1131.

Melloni, E., Minafra, R., Salamino, F. & Pontremoli, S. 2000. Properties and intracellular localization of calpain activator protein. Biochem. Biophys. Res. Commun. 272, 472-476.

Mendelsohn, C.L., Wimmer, E. & Racaniello, V.R. 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell 56, 855-865.

Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E. & Pontremoli, S. 1996. Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localization. FEBS Lett. 392, 11-15.

Milstone, A.M., Petrella, J., Sanchez, M.D., Mahmud, M., Whitbeck, J.C. & Bergelson, J.M. 2005. Interaction with coxsackievirus and adenovirus receptor, but not with decay-accelerating factor (DAF), induces A-particle formation in a DAF-binding coxsackievirus B3 isolate. J. Virol. 79, 655-660.

Mineo, C., Ying, Y.S., Chapline, C., Jaken, S. & Anderson, R.G. 1998. Targeting of protein kinase Calpha to caveolae. J. Cell Biol. 141, 601-610.

Miranti, C.K. & Brugge, J.S. 2002. Sensing the environment: a historical perspective on integrin signal transduction. Nat. Cell Biol. 4, E83-90.

Mirmomeni, M.H., Hughes, P.J. & Stanway, G. 1997. An RNA tertiary structure in the 3' untranslated region of enteroviruses is necessary for efficient

replication. J. Virol. 71, 2363-2370.
Mitra, S.K., Hanson, D.A. & Schlaepfer, D.D. 2005. Focal adhesion kinase: in command and control of cell motility. Nature Rev. Mol. Cell Biol. 6, 56-68.
Miyamoto, S., Akiyama, S.K. & Yamada, K.M. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 267, 883-885.
Moldoveanu, T., Jia, Z. & Davies, P.L. 2004. Calpain activation by cooperative Ca2+ binding at two non-EF-hand sites. J. Biol. Chem. 279, 6106-6114.
Molla, A., Hellen, C.U. & Wimmer, E. 1993. Inhibition of proteolytic activity of poliovirus and rhinovirus 2A proteinases by elastase-specific inhibitors. J. Virol. 67, 4688-4695.
Monier, S., Parton, R.G., Vogel, F., Behlke, J., Henske, A. & Kurzchalia, T.V. 1995. VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. Mol. Biol. Cell 6, 911-927.
Morasco, B.J., Sharma, N., Parilla, J. & Flanegan, J.B. 2003. Poliovirus cre(2C)-dependent synthesis of VPgpUpU is required for positive- but not negative-strand RNA synthesis. J. Virol. 77, 5136-5144.
Morford, L.A., Forrest, K., Logan, B., Overstreet, L.K., Goebel, J., Brooks, W.H. & Roszman, T.L. 2002. Calpain II colocalizes with detergent-insoluble rafts on human and Jurkat T-cells. Biochem. Biophys. Res. Commun. 295, 540-546.
Moro, L., Dolce, L., Cabodi, S., Bergatto, E., Boeri Erba, E., Smeriglio, M., Turco, E., Retta, S.F., Giuffrida, M.G., Venturino, M., Godovac-Zimmermann, J., Conti, A., Schaefer, E., Beguinot, L., Tacchetti, C., Gaggini, P., Silengo, L., Tarone, G. & Defilippi, P. 2002. Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J. Biol. Chem. 277, 9405-9414.
Moscufo, N., Yafal, A.G., Rogove, A., Hogle, J. & Chow, M. 1993. A mutation in VP4 defines a new step in the late stages of cell entry by poliovirus. J. Virol. 67, 5075-5078.
Mossessova, E., Corpina, R.A. & Goldberg, J. 2003. Crystal structure of ARF1*Sec7 complexed with Brefeldin A and its implications for the guanine nucleotide exchange mechanism. Mol. Cell 12, 1403-1411.
Mostafavi-Pour, Z., Askari, J.A., Parkinson, S.J., Parker, P.J., Ng, T.T. & Humphries, M.J. 2003. Integrin-specific signaling pathways controlling focal adhesion formation and cell migration. J. Cell Biol. 161, 155-167.
Mu, F.T., Callaghan, J.M., Steele-Mortimer, O., Stenmark, H., Parton, R.G., Campbell, P.L., McCluskey, J., Yeo, J.P., Tock, E.P. & Toh, B.H. 1995. EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. J. Biol. Chem. 270, 13503-13511.
Muckelbauer, J.K., Kremer, M., Minor, I., Diana, G., Dutko, F.J., Groarke, J., Pevear, D.C. & Rossmann, M.G. 1995. The structure of coxsackievirus B3 at 3.5 A resolution. Structure 3, 653-667.
Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V. & Simons, K. 1995. VIP21/caveolin is a cholesterol-binding protein. Proc. Natl. Acad. Sci. USA 92, 10339-10343.

Murray, K.E. & Barton, D.J. 2003. Poliovirus CRE-dependent VPg uridylylation is required for positive-strand RNA synthesis but not for negative-strand RNA synthesis. J. Virol. 77, 4739-4750.

Mäkelä, M., Vaarala, O., Hermann, R., Salminen, K., Vahlberg, T., Veijola, R., Hyoty, H., Knip, M., Simell, O. & Ilonen, J. 2006. Enteral virus infections in early childhood and an enhanced type 1 diabetes-associated antibody response to dietary insulin. J. Autoimmun. 27, 54-61.

Nelsen-Salz, B., Eggers, H.J. & Zimmermann, H. 1999. Integrin alpha(v)beta3 (vitronectin receptor) is a candidate receptor for the virulent echovirus 9 strain Barty. J. Gen. Virol. 80, 2311-2313.

Ng, T., Shima, D., Squire, A., Bastiaens, P.I., Gschmeissner, S., Humphries, M.J. & Parker, P.J. 1999a. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. EMBO J. 18, 3909-3923.

Ng, T., Squire, A., Hansra, G., Bornancin, F., Prevostel, C., Hanby, A., Harris, W., Barnes, D., Schmidt, S., Mellor, H., Bastiaens, P.I. & Parker, P.J. 1999b. Imaging protein kinase Calpha activation in cells. Science 283, 2085-2089.

Nichols, B.J. 2002. A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. Nat. Cell. Biol. 4, 374-378.

Nobes, C.D. & Hall, A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53-62.

Nobes, C.D., Hawkins, P., Stephens, L. & Hall, A. 1995. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J. Cell. Sci. 108 225-233.

Novak, J.E. & Kirkegaard, K. 1994. Coupling between genome translation and replication in an RNA virus. Genes Dev. 8, 1726-1737.

Nugent, C.I., Johnson, K.L., Sarnow, P. & Kirkegaard, K. 1999. Functional coupling between replication and packaging of poliovirus replicon RNA. J. Virol. 73, 427-435.

Nuzzi, P.A., Senetar, M.A. & Huttenlocher, A. 2007. Asymmetric localization of calpain 2 during neutrophil chemotaxis. Mol. Biol. Cell 18, 795-805.

O'Donnell, V., LaRocco, M., Duque, H. & Baxt, B. 2005. Analysis of foot-and-mouth disease virus internalization events in cultured cells. J. Virol. 79, 8506-8518.

Ochoa, G.C., Slepnev, V.I., Neff, L., Ringstad, N., Takei, K., Daniell, L., Kim, W., Cao, H., McNiven, M., Baron, R. & De Camilli, P. 2000. A functional link between dynamin and the actin cytoskeleton at podosomes. J. Cell Biol. 150, 377-389.

Oh, P., McIntosh, D.P. & Schnitzer, J.E. 1998. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol. 141, 101-114.

Oh, P. & Schnitzer, J.E. 1999. Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification. J. Biol. Chem. 274, 23144-23154.

Ohman, T., King, S.L., Krithivas, A., Cunningham, J., Dickeson, S.K., Santoro, S.A. & Bergelson, J.M. 2001. Echoviruses 1 and 8 are closely related genetically, and bind to similar determinants within the VLA-2 I domain. Virus Res. 76,

1-8.

Olson, N.H., Kolatkar, P.R., Oliveira, M.A., Cheng, R.H., Greve, J.M., McClelland, A., Baker, T.S. & Rossmann, M.G. 1993. Structure of a human rhinovirus complexed with its receptor molecule. Proc. Natl. Acad. Sci. USA 90, 507-511.

Pankov, R., Cukierman, E., Katz, B.Z., Matsumoto, K., Lin, D.C., Lin, S., Hahn, C. & Yamada, K.M. 2000. Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J. Cell Biol. 148, 1075-1090.

Parsons, M., Keppler, M.D., Kline, A., Messent, A., Humphries, M.J., Gilchrist, R., Hart, I.R., Quittau-Prevostel, C., Hughes, W.E., Parker, P.J. & Ng, T. 2002. Site-directed perturbation of protein kinase C- integrin interaction blocks carcinoma cell chemotaxis. Mol. Cell. Biol. 22, 5897-5911.

Parton, R.G., Joggerst, B. & Simons, K. 1994. Regulated internalization of caveolae. J. Cell Biol. 127, 1199-1215.

Parton, R.G. & Simons, K. 2007. The multiple faces of caveolae. Nature Rev. Mol. Cell. Biol. 8, 185-194.

Pathak, H.B., Arnold, J.J., Wiegand, P.N., Hargittai, M.R. & Cameron, C.E. 2007. Picornavirus genome replication: assembly and organization of the VPg uridylylation ribonucleoprotein (initiation) complex. J. Biol. Chem. 282, 16202-16213.

Paul, A.V., Rieder, E., Kim, D.W., van Boom, J.H. & Wimmer, E. 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J. Virol. 74, 10359-10370.

Paul, A.V., van Boom, J.H., Filippov, D. & Wimmer, E. 1998. Protein-primed RNA synthesis by purified poliovirus RNA polymerase. Nature 393, 280-284.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E. & Zerial, M. 2005. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature 436, 78-86.

Pelkmans, L., Kartenbeck, J. & Helenius, A. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat. Cell Biol. 3, 473-483.

Pelkmans, L., Puntener, D. & Helenius, A. 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science 296, 535-539.

Pelletier, J. & Sonenberg, N. 1989. Internal binding of eucaryotic ribosomes on poliovirus RNA: translation in HeLa cell extracts. J. Virol. 63, 441-444.

Pellinen, T., Arjonen, A., Vuoriluoto, K., Kallio, K., Fransen, J.A. & Ivaska, J. 2006. Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. J. Cell Biol. 173, 767-780.

Pellinen, T. & Ivaska, J. 2006. Integrin traffic. J. Cell. Sci. 119, 3723-3731.

Perez, L. & Carrasco, L. 1993. Entry of poliovirus into cells does not require a low-pH step. J. Virol. 67, 4543-4548.

Pfaff, M., Du, X. & Ginsberg, M.H. 1999. Calpain cleavage of integrin beta cytoplasmic domains. FEBS Lett. 460, 17-22.

Phelps, D.K. & Post, C.B. 1995. A novel basis of capsid stabilization by antiviral

compounds. J. Mol. Biol. 254, 544-551.
Pietiäinen, V., Huttunen, P. & Hyypiä, T. 2000. Effects of echovirus 1 infection on cellular gene expression. Virology 276, 243-250.
Pilipenko, E.V., Maslova, S.V., Sinyakov, A.N. & Agol, V.I. 1992a. Towards identification of cis-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. Nucleic. Acids. Res. 20, 1739-1745.
Pilipenko, E.V., Maslova, S.V., Sinyakov, A.N. & Agol, V.I. 1992b. Towards identification of cis-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. Nucleic Acids Res 20, 1739-1745.
Pinton, P., Pozzan, T. & Rizzuto, R. 1998. The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J. 17, 5298-5308.
Piper, R.C. & Luzio, J.P. 2001. Late endosomes: sorting and partitioning in multivesicular bodies. Traffic 2, 612-621.
Pol, A., Luetterforst, R., Lindsay, M., Heino, S., Ikonen, E. & Parton, R.G. 2001. A caveolin dominant negative mutant associates with lipid bodies and induces intracellular cholesterol imbalance. J. Cell Biol. 152, 1057-1070.
Powelka, A.M., Sun, J., Li, J., Gao, M., Shaw, L.M., Sonnenberg, A. & Hsu, V.W. 2004. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic 5, 20-36.
Racoosin, E.L. & Swanson, J.A. 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. J. Cell Biol. 121, 1011-1020.
Randazzo, P.A. & Hirsch, D.S. 2004. Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. Cell. Signal. 16, 401-413.
Ravanti, L., Heino, J., Lopez-Otin, C. & Kahari, V.M. 1999. Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. J. Biol. Chem. 274, 2446-2455.
Razeghi, P., Volpini, K.C., Wang, M.E., Youker, K.A., Stepkowski, S. & Taegtmeyer, H. 2007. Mechanical unloading of the heart activates the calpain system. J. Mol. Cell Cardiol. 42, 449-452.
Rieder, E., Paul, A.V., Kim, D.W., van Boom, J.H. & Wimmer, E. 2000. Genetic and biochemical studies of poliovirus cis-acting replication element cre in relation to VPg uridylylation. J. Virol. 74, 10371-10380.
Roehl, H.H. & Semler, B.L. 1995. Poliovirus infection enhances the formation of two ribonucleoprotein complexes at the 3' end of viral negative-strand RNA. J. Virol. 69, 2954-2961.
Roivainen, M. 2006. Enteroviruses: new findings on the role of enteroviruses in type 1 diabetes. Int. J. Biochem. Cell. Biol. 38, 721-725.
Roivainen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J. & Hyypiä, T. 1994. Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor. Virology 203, 357-365.
Rojo, M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R.G. & Gruenberg, J. 1997. Involvement of the transmembrane protein p23 in biosynthetic protein transport. J. Cell Biol. 139, 1119-1135.

Rossmann, M.G. 1989. The canyon hypothesis. Hiding the host cell receptor attachment site on a viral surface from immune surveillance. J. Biol. Chem. 264, 14587-14590.

Rossmann, M.G., Arnold, E., Erickson, J.W., Frankenberger, E.A., Griffith, J.P., Hecht, H.J., Johnson, J.E., Kamer, G., Luo, M., Mosser, A.G. & et al. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature 317, 145-153.

Rossmann, M.G., He, Y. & Kuhn, R.J. 2002. Picornavirus-receptor interactions. Trends. Microbiol. 10, 324-331.

Roy, S., Luetterforst, R., Harding, A., Apolloni, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J.F. & Parton, R.G. 1999. Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. Nat. Cell Biol. 1, 98-105.

Ruoslahti, E. & Pierschbacher, M.D. 1987. New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

Sabharanjak, S., Sharma, P., Parton, R.G. & Mayor, S. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev. Cell 2, 411-423.

Saido, T.C., Shibata, M., Takenawa, T., Murofushi, H. & Suzuki, K. 1992. Positive regulation of mu-calpain action by polyphosphoinositides. J. Biol. Chem. 267, 24585-24590.

Salonen, A., Ahola, T. & Kääriäinen, L. 2005. Viral RNA replication in association with cellular membranes. Curr. Top. Microbiol. Immunol. 285, 139-173.

Sasaki, J. & Taniguchi, K. 2003. The 5'-end sequence of the genome of Aichi virus, a picornavirus, contains an element critical for viral RNA encapsidation. J. Virol. 77, 3542-3548.

Sato, K. & Kawashima, S. 2001. Calpain function in the modulation of signal transduction molecules. Biol. Chem. 382, 743-751.

Savina, A., Fader, C.M., Damiani, M.T. & Colombo, M.I. 2005. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. Traffic 6, 131-143.

Schaller, M.D., Hildebrand, J.D., Shannon, J.D., Fox, J.W., Vines, R.R. & Parsons, J.T. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol. Cell. Biol. 14, 1680-1688.

Schlegel, A., Giddings, T.H., Jr., Ladinsky, M.S. & Kirkegaard, K. 1996. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J. Virol. 70, 6576-6588.

Schoenwaelder, S.M., Yuan, Y., Cooray, P., Salem, H.H. & Jackson, S.P. 1997. Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin alphaIIbbeta3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. J. Biol. Chem. 272, 1694-1702.

Shafren, D.R. 1998. Viral cell entry induced by cross-linked decay-accelerating factor. J. Virol. 72, 9407-9412.

Shao, H., Chou, J., Baty, C.J., Burke, N.A., Watkins, S.C., Stolz, D.B. & Wells, A. 2006. Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. Mol. Cell. Biol. 26, 5481-5496.

Shepard, D.A., Heinz, B.A. & Rueckert, R.R. 1993. WIN 52035-2 inhibits both attachment and eclipse of human rhinovirus 14. J. Virol. 67, 2245-2254.

Shimaoka, M., Xiao, T., Liu, J.H., Yang, Y., Dong, Y., Jun, C.D., McCormack, A., Zhang, R., Joachimiak, A., Takagi, J., Wang, J.H. & Springer, T.A. 2003. Structures of the alpha L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. Cell 112, 99-111.

Silberstein, E., Dveksler, G. & Kaplan, G.G. 2001. Neutralization of hepatitis A virus (HAV) by an immunoadhesin containing the cysteine-rich region of HAV cellular receptor-1. J. Virol. 75, 717-725.

Simons, K. & Ikonen, E. 1997. Functional rafts in cell membranes. Nature 387, 569-572.

Smart, E.J., Foster, D.C., Ying, Y.S., Kamen, B.A. & Anderson, R.G. 1994. Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae. J. Cell Biol. 124, 307-313.

Smith, A.E. & Helenius, A. 2004. How viruses enter animal cells. Science 304, 237-242.

Smith, T.J., Kremer, M.J., Luo, M., Vriend, G., Arnold, E., Kamer, G., Rossmann, M.G., McKinlay, M.A., Diana, G.D. & Otto, M.J. 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. Science 233, 1286-1293.

Snyers, L., Zwickl, H. & Blaas, D. 2003. Human rhinovirus type 2 is internalized by clathrin-mediated endocytosis. J. Virol. 77, 5360-5369.

Sorimachi, H. & Suzuki, K. 2001. The structure of calpain. J. Biochem. 129, 653-664.

Stanway, G. 1990. Structure, function and evolution of picornaviruses. J. Gen. Virol. 71 2483-2501.

Stanway, G. & Hyypiä, T. 1999. Parechoviruses. J. Virol. 73, 5249-5254.

Steegmaier, M., Yang, B., Yoo, J.S., Huang, B., Shen, M., Yu, S., Luo, Y. & Scheller, R.H. 1998. Three novel proteins of the syntaxin/SNAP-25 family. J. Biol. Chem. 273, 34171-34179.

Stoneley, M. & Willis, A.E. 2004. Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. Oncogene 23, 3200-3207.

Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H., Suzuki, K. & Bode, W. 2000. The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. USA 97, 588-592.

Stuart, A.D., Eustace, H.E., McKee, T.A. & Brown, T.D. 2002. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. J. Virol. 76, 9307-9322.

Suomalainen, M. & Garoff, H. 1994. Incorporation of homologous and heterologous proteins into the envelope of Moloney murine leukemia virus. J. Virol. 68, 4879-4889.

Suzuki, K., Hata, S., Kawabata, Y. & Sorimachi, H. 2004. Structure, activation, and biology of calpain. Diabetes 53 Suppl 1, S12-18.

Suzuki, K. & Sorimachi, H. 1998. A novel aspect of calpain activation. FEBS Lett. 433, 1-4.

Swanson, J.A. & Watts, C. 1995. Macropinocytosis. Trends Cell. Biol. 5, 424-428.

Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L. & Helenius, A. 2005. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J. Cell Biol. 170, 769-779.

Takagi, J., Petre, B.M., Walz, T. & Springer, T.A. 2002. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 110, 599-511.

Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., Maki, M. & Saido, T.C. 2005. Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. J. Biol. Chem. 280, 16175-16184.

Tauriainen, S., Martiskainen, M., Oikarinen, S., Lönnrot, M., Viskari, H., Ilonen, J., Simell, O., Knip, M. & Hyöty, H. 2007. Human parechovirus 1 infections in young children--no association with type 1 diabetes. J. Med. Virol. 79, 457-462.

Teasdale, R.D., Loci, D., Houghton, F., Karlsson, L. & Gleeson, P.A. 2001. A large family of endosome-localized proteins related to sorting nexin 1. Biochem. J. 358, 7-16.

Teranishi, F., Liu, Z.Q., Kunimatsu, M., Imai, K., Takeyama, H., Manabe, T., Sasaki, M. & Okamoto, T. 2003. Calpain is involved in the HIV replication from the latently infected OM10.1 cells. Biochem. Biophys. Res. Commun. 303, 940-946.

Thompson, K.M. & Tebbens, R.J. 2007. Eradication versus control for poliomyelitis: an economic analysis. Lancet 369, 1363-1371.

Thorne, R.F., Marshall, J.F., Shafren, D.R., Gibson, P.G., Hart, I.R. & Burns, G.F. 2000. The integrins alpha3beta1 and alpha6beta1 physically and functionally associate with CD36 in human melanoma cells. Requirement for the extracellular domain OF CD36. J. Biol. Chem. 275, 35264-35275.

Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilagyi, A., Banoczi, Z., Hudecz, F. & Friedrich, P. 2004. On the sequential determinants of calpain cleavage. J. Biol. Chem. 279, 20775-20785.

Tompa, P., Emori, Y., Sorimachi, H., Suzuki, K. & Friedrich, P. 2001. Domain III of calpain is a ca2+-regulated phospholipid-binding domain. Biochem. Biophys. Res. Commun. 280, 1333-1339.

Tompa, P., Mucsi, Z., Orosz, G. & Friedrich, P. 2002. Calpastatin subdomains A and C are activators of calpain. J. Biol. Chem. 277, 9022-9026.

Torgersen, M.L., Skretting, G., van Deurs, B. & Sandvig, K. 2001. Internalization of cholera toxin by different endocytic mechanisms. J. Cell. Sci. 114, 3737-3747.

Tosteson, M.T. & Chow, M. 1997. Characterization of the ion channels formed by poliovirus in planar lipid membranes. J. Virol. 71, 507-511.

Tosteson, M.T., Wang, H., Naumov, A. & Chow, M. 2004. Poliovirus binding to its receptor in lipid bilayers results in particle-specific, temperature-sensitive channels. J. Gen. Virol. 85, 1581-1589.

Toyoda, H., Nicklin, M.J., Murray, M.G., Anderson, C.W., Dunn, J.J., Studier, F.W. & Wimmer, E. 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45, 761-770.

Traub, L.M. 2003. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic

cargo selection. J. Cell Biol. 163, 203-208.
Triantafilou, K. & Triantafilou, M. 2001. A biochemical approach reveals cell-surface molecules utilised by Picornaviridae: Human Parechovirus 1 and Echovirus 1. J. Cell. Biochem. 80, 373-381.
Triantafilou, K. & Triantafilou, M. 2003. Lipid raft microdomains: key sites for Coxsackievirus A9 infectious cycle. Virology 317, 128-135.
Triantafilou, K. & Triantafilou, M. 2004. Lipid-raft-dependent Coxsackievirus B4 internalization and rapid targeting to the Golgi. Virology 326, 6-19.
Triantafilou, K., Triantafilou, M., Takada, Y. & Fernandez, N. 2000. Human parechovirus 1 utilizes integrins alphavbeta3 and alphavbeta1 as receptors. J. Virol. 74, 5856-5862.
Trigatti, B.L., Anderson, R.G. & Gerber, G.E. 1999. Identification of caveolin-1 as a fatty acid binding protein. Biochem. Biophys. Res. Commun. 255, 34-39.
Trono, D., Pelletier, J., Sonenberg, N. & Baltimore, D. 1988. Translation in mammalian cells of a gene linked to the poliovirus 5' noncoding region. Science 241, 445-448.
Tsang, S.K., McDermott, B.M., Racaniello, V.R. & Hogle, J.M. 2001. Kinetic analysis of the effect of poliovirus receptor on viral uncoating: the receptor as a catalyst. J. Virol. 75, 4984-4989.
van Kuppeveld, F.J., Hoenderop, J.G., Smeets, R.L., Willems, P.H., Dijkman, H.B., Galama, J.M. & Melchers, W.J. 1997. Coxsackievirus protein 2B modifies endoplasmic reticulum membrane and plasma membrane permeability and facilitates virus release. EMBO J. 16, 3519-3532.
van Ooij, M.J., Vogt, D.A., Paul, A., Castro, C., Kuijpers, J., van Kuppeveld, F.J., Cameron, C.E., Wimmer, E., Andino, R. & Melchers, W.J. 2006. Structural and functional characterization of the coxsackievirus B3 CRE(2C): role of CRE(2C) in negative- and positive-strand RNA synthesis. J. Gen. Virol. 87, 103-113.
Wang, J. 2002. Protein recognition by cell surface receptors: physiological receptors versus virus interactions. Trends Biochem. Sci. 27, 122-126.
Ward, T., Powell, R.M., Pipkin, P.A., Evans, D.J., Minor, P.D. & Almond, J.W. 1998. Role for beta2-microglobulin in echovirus infection of rhabdomyosarcoma cells. J. Virol. 72, 5360-5365.
Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E. & Giancotti, F.G. 1996. The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell 87, 733-743.
Wary, K.K., Mariotti, A., Zurzolo, C. & Giancotti, F.G. 1998. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 94, 625-634.
Way, M. & Parton, R.G. 1995. M-caveolin, a muscle-specific caveolin-related protein. FEBS Lett. 376, 108-112.
Wehrle-Haller, B. & Imhof, B.A. 2003. Integrin-dependent pathologies. J. Pathol. 200, 481-487.
Wei, Y., Yang, X., Liu, Q., Wilkins, J.A. & Chapman, H.A. 1999. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signaling. J. Cell Biol. 144, 1285-1294.
Wells, V.R., Plotch, S.J. & DeStefano, J.J. 2001. Determination of the mutation rate of

poliovirus RNA-dependent RNA polymerase. Virus Res. 74, 119-132.
Verdaguer, N., Blaas, D. & Fita, I. 2000. Structure of human rhinovirus serotype 2 (HRV2). J. Mol. Biol. 300, 1179-1194.
West, M.A., Bretscher, M.S. & Watts, C. 1989. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. J. Cell Biol. 109, 2731-2739.
West, M.A., Prescott, A.R., Eskelinen, E.L., Ridley, A.J. & Watts, C. 2000. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. Curr. Biol. 10, 839-848.
Westhoff, M.A., Serrels, B., Fincham, V.J., Frame, M.C. & Carragher, N.O. 2004. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. Mol. Cell. Biol. 24, 8113-8133.
Wetz, K. & Kucinski, T. 1991. Influence of different ionic and pH environments on structural alterations of poliovirus and their possible relation to virus uncoating. J. Gen. Virol. 72, 2541-2544.
Williams, C.H., Kajander, T., Hyypiä, T., Jackson, T., Sheppard, D. & Stanway, G. 2004. Integrin alpha v beta 6 is an RGD-dependent receptor for coxsackievirus A9. J. Virol. 78, 6967-6973.
Wimmer, E., Hellen, C.U. & Cao, X. 1993. Genetics of poliovirus. Annu. Rev. Genet. 27, 353-436.
Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E. & Qin, J. 2002. A structural mechanism of integrin alpha(IIb)beta(3) "inside-out" activation as regulated by its cytoplasmic face. Cell 110, 587-597.
Wong, J., Zhang, J., Si, X., Gao, G. & Luo, H. 2007. Inhibition of the extracellular signal-regulated kinase signaling pathway is correlated with proteasome inhibitor suppression of coxsackievirus replication. Biochem. Biophys. Res. Commun. 358, 903-907.
Wozniak, M.A., Modzelewska, K., Kwong, L. & Keely, P.J. 2004. Focal adhesion regulation of cell behavior. Biochim. Biophys. Acta 1692, 103-119.
Vuorinen, T., Vainionpää, R., Kettinen, H. & Hyypiä, T. 1994. Coxsackievirus B3 infection in human leukocytes and lymphoid cell lines. Blood 84, 823-829.
Xiang, W., Harris, K.S., Alexander, L. & Wimmer, E. 1995. Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J. Virol. 69, 3658-3667.
Xiao, C., Bator, C.M., Bowman, V.D., Rieder, E., He, Y., Hebert, B., Bella, J., Baker, T.S., Wimmer, E., Kuhn, R.J. & Rossmann, M.G. 2001. Interaction of coxsackievirus A21 with its cellular receptor, ICAM-1. J. Virol. 75, 2444-2451.
Xiao, T., Takagi, J., Coller, B.S., Wang, J.H. & Springer, T.A. 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432, 59-67.
Xing, L., Huhtala, M., Pietiäinen, V., Käpylä, J., Vuorinen, K., Marjomäki, V., Heino, J., Johnson, M.S., Hyypiä, T. & Cheng, R.H. 2004. Structural and functional analysis of integrin alpha2I domain interaction with echovirus 1. J. Biol. Chem. 279, 11632-11638.
Xiong, J.P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D.L., Joachimiak, A., Goodman, S.L. & Arnaout, M.A. 2001. Crystal structure of the

extracellular segment of integrin alpha Vbeta3. Science 294, 339-345.

Xu, L. & Deng, X. 2006. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. J. Biol. Chem. 281, 35567-35575.

Yamada, K.M. & Even-Ram, S. 2002. Integrin regulation of growth factor receptors. Nat. Cell Biol. 4, E75-76.

Yan, B., Calderwood, D.A., Yaspan, B. & Ginsberg, M.H. 2001. Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J. Biol. Chem. 276, 28164-28170.

Zamir, E. & Geiger, B. 2001. Molecular complexity and dynamics of cell-matrix adhesions. J. Cell. Sci. 114, 3583-3590.

Zatz, M. & Starling, A. 2005. Calpains and disease. N. Eng. J. Med. 352, 2413-2423.

Zhang, X.A., Kazarov, A.R., Yang, X., Bontrager, A.L., Stipp, C.S. & Hemler, M.E. 2002. Function of the tetraspanin CD151-alpha6beta1 integrin complex during cellular morphogenesis. Mol. Biol. Cell 13, 1-11.

Zhou, L., Lin, X., Green, T.J., Lipton, H.L. & Luo, M. 1997. Role of sialyloligosaccharide binding in Theiler's virus persistence. J. Virol. 71, 9701-9712.

Ziegler, T., Waris, M., Rautiainen, M. & Arstila, P. 1988. Herpes simplex virus detection by macroscopic reading after overnight incubation and immunoperoxidase staining. J. Clin. Microbiol. 26, 2013-2017.