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Virus interaction with the apical junctional complex

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1. ABSTRACT

In order to infect pathogens must breach the epithelial barriers that separate the organism from the external environment or that cover the internal cavities and ducts of the body. Epithelia seal the passage through the paracellular pathway with the apical junctional complex integrated by tight and adherens junctions. In this review we describe how viruses like coxsackie, swine vesicular disease virus, adenovirus, reovirus, feline calcivirus, herpes viruses 1 and 2, pseudorabies, bovine herpes virus 1, poliovirus and hepatitis C use as cellular receptors integral proteins present at the AJC of epithelial cells. Interaction with these proteins contributes in a significant manner in defining the particular tropism of each virus. Besides these proteins, viruses exhibit a wide range of cellular co-receptors among which proteins present in the basolateral cell surface like integrins are often found. Therefore targeting proteins of the AJC constitutes a strategy that might allow viruses to bypass the physical barrier that blocks their access to receptors expressed on the basolateral surface of epithelial cells.

2. INTRODUCTION

One of the distinctive features of epithelial cells is the polarization of their plasma membrane into the apical and basolateral surfaces, each characterized for presenting a distinct morphological and biochemical composition. The apical membrane faces the lumen of internal cavities and ducts or in the case of the skin it is in contact with the air or water that surrounds the body, whereas the basolateral membrane faces the internal milieu of the organism (1).

In multicellular organisms the compartments (e.g. stomach, uterus, urinary bladder) and ducts (e.g. blood vessels, kidney tubules, intestine) of the body maintain substances of diverse composition (e.g. milk, urine, bile, blood, stools) separated from the internal milieu. To attain this purpose epithelial cells strictly regulate the transit of ions and molecules from and into these compartments (2,3).

Cell-cell adhesion starts at the tip of filopodia protruding from neighboring cells, then progresses to the formation of wider areas of contact in lamellipodia and ends up with the establishment of a junctional complex located at the uppermost portion of the lateral membrane that like a belt surrounds all epithelial cells (4). This apical junctional complex (AJC) is integrated by the tight (TJ) and adherens junctions (AJ). AJ initiate cell-cell contacts and for such a purpose employ Ca^{2+} dependent proteins named cadherins and other molecules like nectin and nectin-like (necl) proteins that belong to the immunoglobulin superfamily (5), as well as a set of cytoplasmic adaptor proteins that includes catenins, afadin, vinculin and alpha-actinin. TJs are located immediately above AJ and function as a gate that regulates by size and charge the passage of ions and molecules through the paracellular pathway, and as a fence that blocks the movement of lipids and proteins within the membrane from the apical to the basolateral surface and vice-versa (6). TJs are established once nectins start recruiting to the apical side of AJs, other proteins of the immunoglobulin superfamily named junctional adhesion molecules (JAMs). This results in the posterior membrane recruitment of other integral proteins like claudins and occludin and on the concentration on this region of several adaptor molecules such as the ZO proteins and cingulin, giving rise to the formation of TJs as a cell adhesion entity separate from the AJ (7,8,9,10).

The first line of defense of the body against pathogens is the skin and the epithelial sheets that cover the cavities and ducts of the organism (11). Therefore it is no surprise to find that most viral infections begin when viruses interact with the skin or the epithelia of the respiratory, genital or gastrointestinal (GI) tracts. While some viral infections are limited to these epithelia (e.g. rotavirus, influenza), other viruses traverse the epithelial sheets and spread inside the body to other organs (e.g. polio, HIV).

There are two main entry pathways for viruses into epithelial cells. The first is the endocytic route where virus penetrates the cell either by clathrin mediated endocytosis or by caveolae. The second route is the non-endocytic pathway and involves fusion of the viral particle with the plasma membrane, or from inside an endosome (12).

All these strategies however start at the molecular level by the association of viral proteins with host cell molecules to which we shall refer to as viral attachment receptors. Identification of such receptors is important for understanding virus tropism, pathogenicity and the mechanisms of virus entry.

Virus receptor function is known to be affected by the organization of the cell membrane. In particular the role of lipid rafts has been studied. Lipid rafts are microdomains in the cell membrane, which are enriched with cholesterol glycolipids and sphingolipids (13). Because of their composition they are resistant to non-ionic detergents and are therefore called detergent resistant membranes (DRMs). The function of lipid rafts in virus entry remains controversial (14). They appear to be important for increasing the local concentration of molecules involved in virus entry (15,16) and some raft forming glycosphingolipids are known to interact directly with viral entry proteins and cellular receptor molecules (17). Additionally it should be mentioned that the TJs are raft like microdomains (18) and as will be described in this review several viruses employ as cell receptors proteins located at the TJ.

Viruses are thought not to use unique viral receptors, but to associate instead to a variety of host cell molecules. Thus viruses mimic the natural ligands of these cell receptors and interfere with their signaling to promote their entry into the organism. Virus-cell attachment is a multi-step process that involves recognition and interaction of various proteins with diverse sequences, structures and cellular functions (19). Among the most important viral attachment receptors are:

1) The carbohydrate chains of proteoglycans and epithelial membrane glycosphingolipids that bind to viral surface proteins, which exhibit several lectin sites.

2) Integrins that concentrate at the basolateral surface of epithelial cells where they mediate interaction with molecules of the extracellular matrix that exhibit particular motifs (e.g. RGD, KGE, DGE, GPR y LDV) which are also found in viral surface proteins (20,21).

3) Cell-cell adhesion molecules. Proteins present at AJs and TJs have in recent years been found to be used by a broad spectrum of viruses to penetrate epithelial cells (Table 1). The use of such proteins implies the disruption of the AJC compromising as a result the integrity of the epithelial barrier. In this review we will describe the nature of the cell adhesion molecules of the AJC used as viral receptors and afterthat we will focus on how these proteins are employed by different viruses for their entry into epithelial cells.

Table 1. Viruses that employ as cell receptors proteins present in the apical junctional complex

Virus	Family	Genus	Viral protein	Cell Receptor	Subcellular Location of Receptor	Viral tropism	Disease
Coxsackie	Picornaviridae	Enterovirus	VP1, VP2 and VP3	CAR	TJ	GI tract, heart, brain pancreas	Bornholm, myocarditis, pericaraditis, aseptic meningitis, Diabetes Mellitus type I
Swine vesicular disease virus	Picornaviridae	Enterovirus	ND	CAR	TJ	GI tract	Swine vesicular disease
Poliovirus	Picornaviridae	Enterovirus	VP1	ncl-5	AJ	GI tract, blood, motor neurons of the spinal cord, brain stem or motor cortex	Poliomelitis
Adenovirus	Adenoviridae	Mastadenovirus	Fiber	CAR	TJ	Respiratory and GI tract	tonsilitis, croup, bronchiolitis, pneumonia, conjunctivitis, gastroenteritis, meningitis, encephalitis, myocarditis
Reovirus	Reoviridae	Orthoreovirus	σ1	JAM-A	TJ	Respiratory and GI tract	Asymptomatic in most mammals including humans
Rotavirus		Rotavirus	Vp8	ND	ND	GI tract	Diarrhea
Feline Calicivirus	Caliciviridae	Vesivirus	ND	JAM-A	TJ	Respiratory tract	Respiratory infections and polyarthritis in felines
HSV-1	Herpesviridae	Simplexvirus	gD	Nectin-1	AJ	Mouth lips, cornea, sensory and autonomic neurons	Lip cold sores, Keratitis
HSV-2	Herpesviridae	Simplexvirus	gD	Nectin-1, nectin-2	AJ	Genitals, sensory and autonomic neurons	Genital Herpes
Pseudorabies	Herpesviridae	Varicellovirus	gD	Nectin-1, nectin-2, ncl-5	AJ	Respiratory tract, cranial nerves, reproductive organs	Pseudorabies or Aujeszky's disease
Bovine Herpes 1	Herpesviridae	Varicellovirus	gD	Nectin-1, ncl-5	AJ	Genitals, eye conjunctive, upper respiratory tract	Rhinotracheitis, Conjunctivitis, Genital infections, Shipping fever
HCV	Flaviviridae	Hepacivirus	ND	Claudins -1, -6 and -9	TJ	Liver, blood	Chronic hepatitis C, Liver cirrhosis, Liver cancer

AJ, adherens junction; TJ, tight junction; ND, non determined.

3. MOLECULES OF THE AJC EMPLOYED BY VIRUSES AS ATTACHMENT RECEPTORS FOR THEIR ENTRY INTO THE ORGANISM

3.1. Junctional adhesion molecules

JAMs belong to the cortical thymocyte Xenopus (CTX) family of proteins that have in the extracellular domain, two immunoglobulin (Ig) like repeats with intramolecular disulfide bonds (22). Proteins belonging to the JAM subfamily have as additional feature a cytoplasmic region that ends with a motif that binds PDZ domains (Figure 2A). The latter are modules of 89-90 residues named after the proteins in which they were initially identified (PSD-95, Dlg and ZO-1) that mediate intermolecular interactions. At the TJ region more than twelve adaptor proteins contain PDZ domains including ZO proteins, MAGIs, Par-3, Par-6, PATJ, PALS-1, AF-6 and MUPP1.

Until now 8 different proteins have been identified as members of the JAM family (Figure 1): JAM-A, JAM-B, JAM-C, CAR, ESAM, JAM4, CLMP and BT-IgSF. These molecules have two or more putative N-linked glycosylation sites in the extracellular region and several consensus phosphorylation sites for PKC, PKA and casein kinase II in their cytoplasmic tails (23,24,25). JAMs A, B and C exhibit a short cytoplasmic tail of 45-50 residues that ends with the type II PDZ binding motif phi-X-phi, (where phi is a hydrophobic amino acid and X any residue), while the others have longer tails of 105 to 165 amino acids ending in the Type I PDZ binding motif S/TXV. An exception is found in CLMP that ends with a QTV motif, which has been suggested to interact with PDZ domains (26). The dendogram in figure 1 reveals how JAMs A, B and C are more closely related among themselves than with the other members of the JAM protein subfamily. This dendogram also illustrates that the relationship of JAM proteins to the nectin and nectin like proteins is more distant than that to the other members of the CTX family.

JAMs interact through their PDZ binding motifs with several proteins present at the AJC. For example, the PDZ binding motif of JAM-A interacts with the PDZ-2 and PDZ-3 domains of ZO-1 (27,28), with the PDZ-9 of MUPP1 (29), with the PDZ domain of afadin (28) and with the PDZ containing proteins Par3 (30) and CASK, a calcium and calmodulin dependent serine protein kinase (31). JAM-A also associates with the TJ proteins cingulin and occludin (27). JAM proteins have been shown to interact with integrins. For example in endothelial cells, JAM-A is a ligand for the leukocyte $beta_2$ integrin LFA1 (32) and displays a cis interaction with integrin $alpha_v beta_3$ expressed by endothelial cells (33,34). Additionally the adhesion of endothelial JAM-B with JAM-C found in the surface of T cells enables a posterior engagement of the leukocyte integrin $alpha_4 beta_1$ to endothelial JAM-B (35).

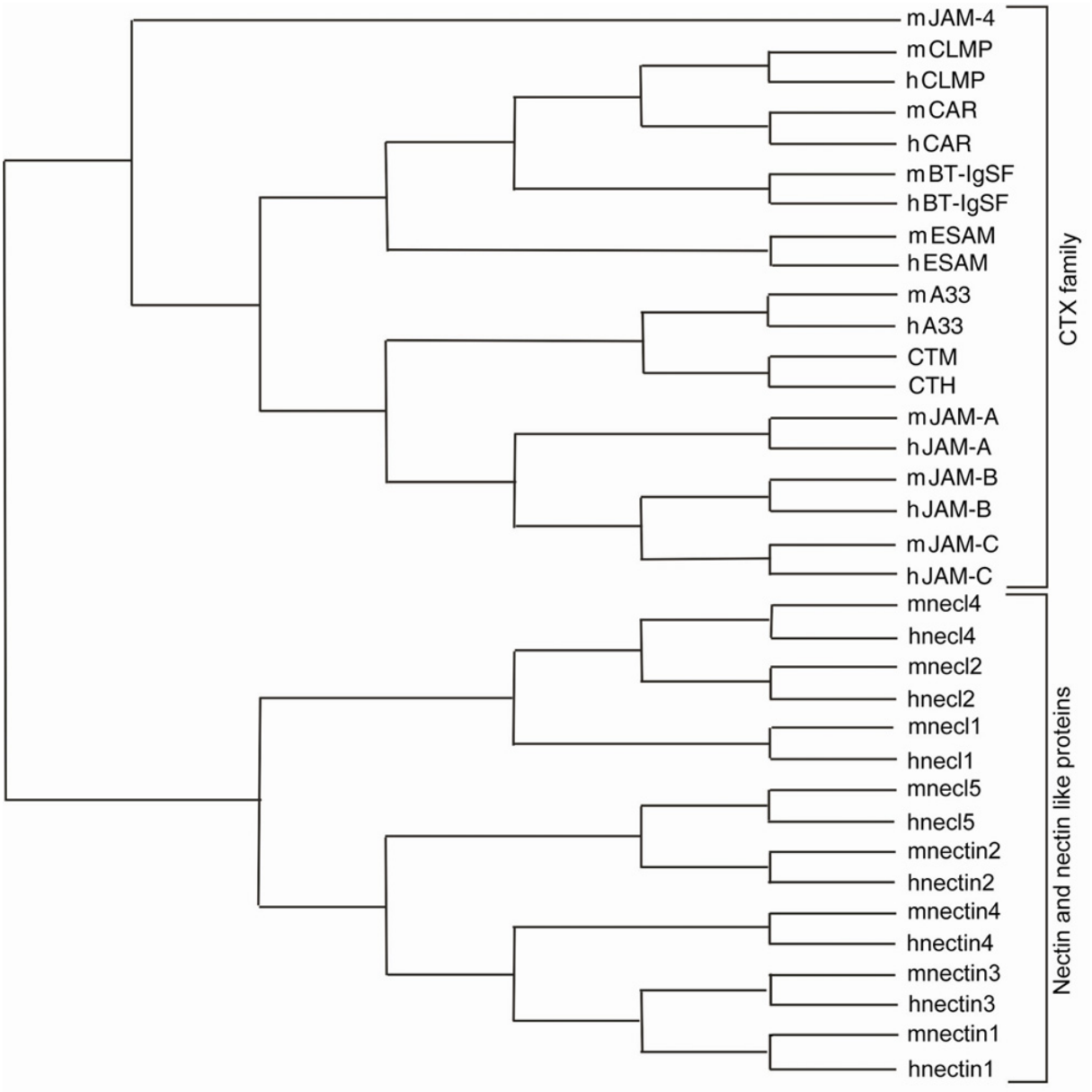

Figure 1. Dendogram representing the relationship between JAM subfamily proteins, CTX family proteins, nectins and nectin like proteins. The tree was constructed using the calculated genetic distances between pairs of members. The sequence data for human and mouse proteins are available from the genbank database under the following accession numbers: hnectin-1, CAA53980; hnectin-2, Q92692; hnectin-3, NP_056295; hnectin-4, NP_112178; hnectl-1, AAD17540; hnectl-2, NP_055148; hnecl-4, AAM60750; hnectl-5, AAA36461; hJAM-2, AAG49022; hJAM-3, AAK27221; hJAM-1, Q9Y624; hCLMP, DAA01139; hBT-IgSF, BAC07546; hCAR, AC51234; hESAM, AAK51065; hA33, AAC50957; mnectin-4, AAL79833; mnectin-3 AAF63685; mnectin-2, P32507; mnectin-1, Q9JKF6; mJAM-3, NP_075766; mJAM-1, AAC32982; mJAM-2, NP_076333; mnectl-1, AAG35584; mnecl-2, AAQ02381; mnectl-4, AAL29692; mnectl-5, CAD91411; mCLMP, AAP15240; mBT-IgSF, BAC07547; mCAR, CAA71368; mESAM, AAK51504; mA33, AAF65818; mJAM4, Q7TSN7; CTH, Q96IQ7 and CTM, Q9Z109

Recently a functional link between JAM-A and $beta_1$ integrin protein but not mRNA levels has been found in epithelial cells revealing that JAM-A regulates cell migration and adhesion through an indirect mechanism that involves post-transcriptional control of $beta_1$ integrin levels (36)

JAMs are among the first proteins to reach the TJ. In mouse embryos they assemble at the cellular borders at the 8 cell stage (7), the same time when the isoform $alpha^-$ of ZO-1 is appearing (10), and earlier than cingulin (16 cell stage) (37), ZO-1 $alpha^+$ (10), claudin-1 (7) and occludin (32 cell stage) (38). In fact JAM arrival to the

membrane appears to tethers the localization of ZO-1, AF6, CASK, occludin and Par-3 to points of cell-cell contact (28,30,31,27,39).

Not all member of the JAM family display the same subcellular localization. For example, JAM-B is located at the lateral membrane (40), JAM-A, CAR, ESAM, JAM-4 and CLMP concentrate at the TJ (41,42,43,44,31,45,46,47), while JAM-C varies its localization depending on the cell type. Thus in canine epithelial MDCK cells JAM-C localizes at TJs (40) while in human intestinal epithelial cells (T84) it concentrates at desmosomes (48).

JAM-A molecules form homodimers in cis through the dimerization motif R (V,I,L)E located in the V type first Ig-like domain (49). This motif is conserved between JAM-A (RVE), JAM-B (RIE) and JAM-C (RLE). The position of the dimerization motif together with the bent conformation of JAM-A due to the linker between the two immunoglobulin domains, results in the formation of homodimers that display the shape of an inverted U. At the intercellular space the JAM-A cis homodimers bind in trans to JAM-A homodimers in the surface of the neighboring cells. Besides these homophilic interactions, JAM proteins are engaged in heterophilic adhesive interactions with other JAM family members (23,50,51) and with other type of cell adhesion molecules such as integrins (52,53,33).

Additionally from their role in TJ assembly, JAMs participate in leukocyte transmigration (54,48,55), platelet activation (56,52), angiogenesis (33) and virus binding (57,58) as will be described in further detail below.

3.2. Claudins

Claudins are the main molecular constituents of TJ filaments observed by freeze fracture. Hence transfection of claudins into cells that lack TJs like fibroblasts, generates the emergence of TJ filaments (59) and in epithelial cell lines, transforms the pre-existent freeze-fracture pattern of TJ strands (60).

Claudins are tetraspan proteins with their amino and carboxyl terminal ends oriented towards the cytoplasm and with two extracellular loops facing the extracellular space (Figure 2B). The first loop is longer than the second one (41 to 55 vs 10 to 21 amino acids), and both exhibit a unique number and type of charged residues. The nature of these residues is crucial as it determines the transepithelial electrical resistance (TER) and the charge selectivity of TJs (61) (62).

The carboxyl terminal end of all claudins contains a PDZ binding motif through which claudins interact with several adaptor proteins of the TJ like ZO-1 (63), ZO-2 (63), MUPP1 (29) and PATJ (64). Claudins are also known to interact with protein kinase WNK4 (65) and the metalloproteinase MMP2 (66).

More than 20 different claudins have been detected in a wide array of epithelia and the particular combination of claudins present in each one determines its TER, ionic selectivity, and pattern of TJ strands (67,68).

3.3. Nectin and nectin like proteins

3.3.1. Nectins

Nectins are Ca^{2+} independent cell adhesion molecules with three extracellular Ig-like domains, a single transmembrane region and a cytoplasmic tail that contains at its carboxyl terminal end a motif that binds afadin (Figure 2A). Initially called PRRs for poliovirus receptor related proteins, these proteins were renamed nectins from the Latin word “necto” meaning to connect, to illustrate their role in cell-cell adhesion (69).

Nectin family has four members, and each member has splicing variants (70). Nectins 1 to 3 are widely expressed in embryos and adult tissues whereas nectin-4 is mainly expressed in human placenta although in mouse it exhibits a broad tissue expression (71).

Nectins initially establish homophilic cis-dimers on the cell surface with their second Ig-like domain. These dimers then interact in trans at cell-cell adhesion sites in a homophilic or heterophilic fashion through their first Ig-like domain (72,73). All nectins can establish homophilic trans interactions, meaning that the nectin on one cell is able to bind to the same molecule on the adjacent cell. Heterophilic trans interactions among nectins exhibit a higher affinity than homophilic trans interactions, and display a particular preference pattern (74). Thus nectin-1 trans interacts with nectins-3 and -4, whereas nectin-2 trans interacts with nectin-3 and CD226/DNAM-1, a molecule expressed in activated T cells and natural killer cells involved in T cell differentiation and proliferation in addition to cytotoxicity (75).

Nectin based cell-cell adhesion plays an essential role in the formation of AJs and TJs and in the establishment of polarity in epithelial cells. For these processes to occur the direct interaction of nectins with afadin and Par-3 is crucial. Afadin is an actin filament binding protein that associates to Rap1, ADIP, LMO7, ZO-1, ponsin and alpha-catenin. Nectins 1 to 3 have a conserved PDZ binding motif (E/AXYV) at their C termini that associates to the PDZ domain of afadin (72,69). Nectin 4 has another C terminal motif that also binds afadin (71). Par-3 is a protein that in association with Par-6 and atypical protein kinase C (aPKC) is required for apico-basal polarization in epithelial cells. Nectin-1 and nectin-3 but not nectin-2 bind the PDZ domain of Par-3 (76). The interaction of nectins with Par-3 promotes the interaction of nectins with afadin (77).

Nectins create the initial cell-cell adhesion and then recruit E-cadherin to the nectin based cell-cell adhesion sites forming AJs. Although the mechanism that regulates nectins and E-cadherins association is not completely understood, it has become clear that both afadin and alpha-catenin are essential for their interaction (78).

Nectins promote cell-cell adhesion by two mechanisms: One involves the recruitment of F actin

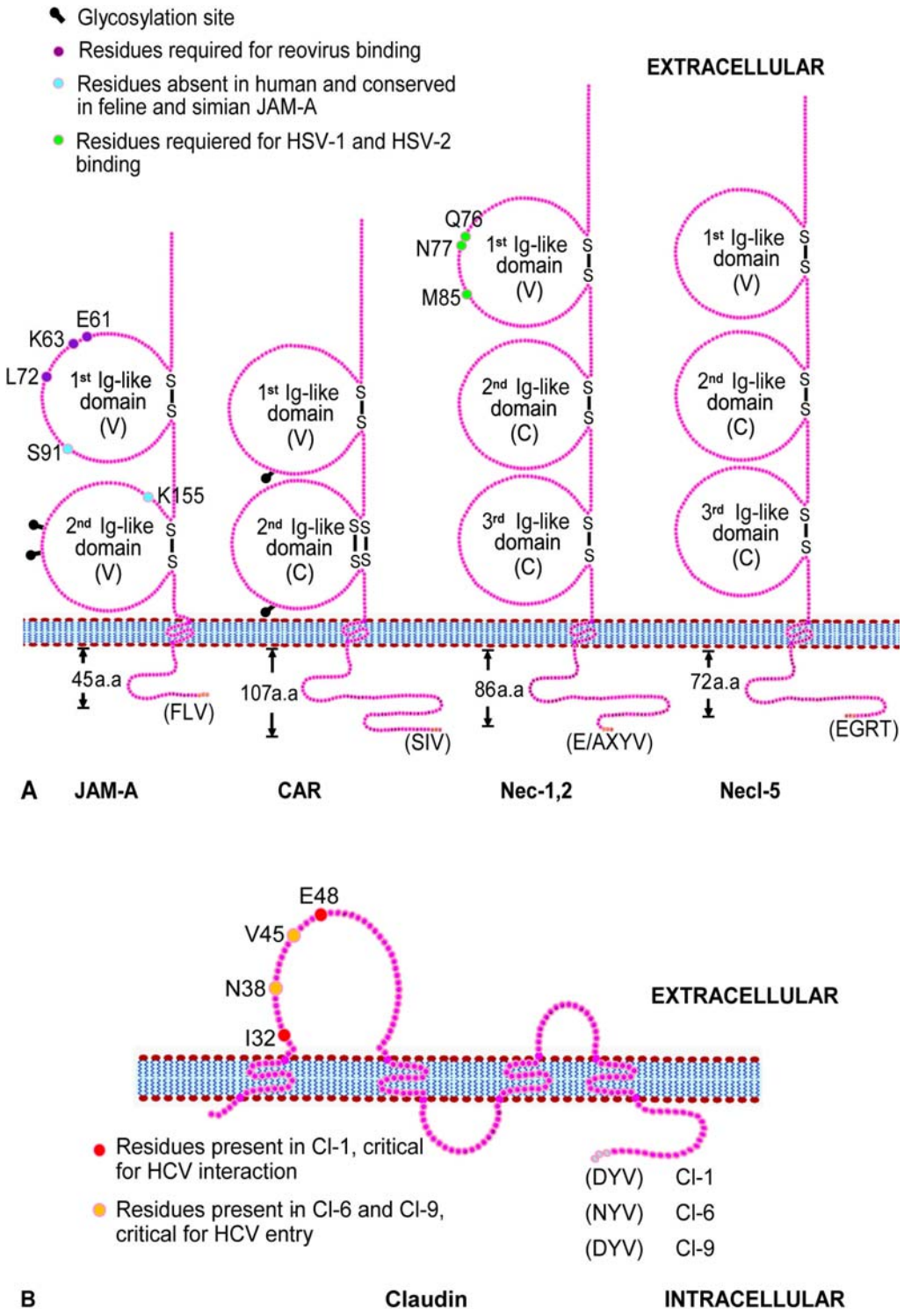

Figure 2. Schematic representation of the integral proteins of the apical junctional complex that function as viral attachment receptors. A) JAM-A, CAR, nectins 1 and 2 and necl-5 are type I proteins of the immunoglobulin superfamily. The first two proteins exhibit two Ig-like repeats while the nectin and necl proteins have three Ig-like domains with intramolecular disulfide bonds. Putative N-linked glycosylation sites are depicted by dots. B) Claudins -1, -6 and -9 are tetraspan proteins with two extracellular loops enriched in charged residues. These entire proteins exhibit at their carboxyl terminal tail a PDZ binding motif whose amino acid residues are here indicated by single letter codes.

binding proteins such as afadin, alpha-catenin, alpha-actinin and vinculin, and the other relies on the activation of Rap1, Cdc42 and Rac small G proteins through activation of c-Src (79,80,81). Activated Rap1 binds to afadin, strengthening the binding of p120 to non-trans interacting E-cadherin, inhibiting its endocytosis and thereby accumulating E-cadherin at cell-cell adhesion sites (82). Activated Cdc42 increases the number of filopodia and activated Rac induces formation of lamellipodia and seals cell-cell adhesion between filopodia like a zipper (83).

Nectins 1 and 3 are able to interact in cis with integrin $alpha_v beta_3$ (84). These interactions start at the primordial nectin based cell-cell adhesion sites and trigger the activation of PKC-FAK-cSrc signaling pathway, necessary for AJ formation (85).

The formation of TJs depends on the previous establishment of nectin based cell-cell adhesion at AJ. Thus claudin polymerization at the uppermost portion of the lateral membrane, above the AJ, requires the presence of dimerized ZO-1 (86), that associates at the AJ to afadin. Hence it is concluded that the formation of TJ strands constituted by polymerized claudins requires a scaffold of nectin, afadin and ZO proteins.

3.3.2. Nectin like proteins

Five molecules with structures similar to those of nectins, but without the ability to directly bind afadin have been named nectin like (necl) proteins. Of them only ncl-5 is known to function as a viral receptor (Figure 2A).

4. DIFFERENT VIRUSES INTERACT WITH PROTEINS OF THE AJC

4.1. Coxsackie, swine vesicular disease virus and Adenovirus interact with CAR, a member of the JAM family

4.1.1. CAR

CAR is the cellular receptor of the coxsackie B and adenovirus, two phylogenetically and structurally unrelated pathogens (87,88). CAR is a 365 amino acid protein that on a SDS-PAGE exhibits a molecular mass higher than expected (46 vs 39 kDa) due to its N-glycosylation at N106 and N201 in the first and second Ig-like domains respectively (89). Loss of glycosylation does not alter the cell surface or junctional localization of CAR but affects both cell-cell adhesion and adenovirus infection (90). Thus CAR mediated adhesion requires at least one site of glycosylation, whereas the lack of glycosylation in the second Ig-like domain paradoxically decreases adenovirus binding and increases infection. An explanation to this conundrum could be that a stronger virus-cell interaction established with glycosylated CAR might retain the adenoviruses at the cell surface resulting in a reduced efficiency of endocytosis.

CAR has a variable (V) type first Ig-like domain and a second constant (C) type Ig-like module (91). The latter domain has an extra pair of cysteines forming a second intradomain disulfide bond (Figure 2A). CAR also exhibits two membrane proximal adjacent cysteines in the cytoplasm that constitute a palmitoylation motif (92). These cysteines are essential for the tumor suppressor activity of CAR but are not critical for adenovirus receptor performance.

The human CAR gene is composed of at least eight exons located on chromosome 21q11.2 (93). The extracellular domain of CAR is encoded by exons I-VI whereas the cytosolic region is encoded by exon VII. A second membranous CAR isoforms named hCAR2 is produced by alternative splicing between an internal splicing site of exon VII and exon VIII (94). Additionally three soluble CAR isoforms that lack the transmembrane domain are generated by alternative splicing (95,96). These soluble proteins are secreted from the cells, bind to the extracellular CAR domain and inhibit coxsackie virus infection, thus indicating that the soluble isoforms of CAR may have a role in the antiviral defense of the host.

CAR is expressed at the TJ of epithelial cells that line the body cavities, and is not present in endothelial cells including those of the brain capillaries (97). CAR expression in different epithelia correlates positively with the maturity of TJs and inversely with permeability (97). For example CAR expression in the nephron is higher in the renal collecting ducts than in the leaky proximal tubules, and CAR levels are much lower in the highly permeable ependymal cells that line the brain ventricles than in the epithelial cells of the choroid plexus that form the cerebro spinal fluid (CSF) barrier.

CAR transfection decreases the permeability of paracellular tracers and increases the TER. The addition of soluble CAR to epithelial cells inhibits the formation of TJs triggered by Ca^{2+} in monolayers previously exposed to the Ca^{2+} chelator EDTA, by competing with the homotypic binding of CAR molecules located in neighboring membranes (98). The ablation of the PDZ binding motif of CAR does not affect adenoviral infection, but alters TJ physiology, as transfection with a CAR mutant lacking the last four amino acids fails to increase TER in epithelial cells (99).

CAR is involved in TJ formation as it recruits the TJ adaptor proteins ZO-1 (98) and MUPP1 (100) to sites of cell-cell contact. MUPP1 is a target for sequestration or degradation of the oncoproteins E4-ORF1 from adenovirus type 9 and E6 from high risk human papillomavirus-18 (101). The interaction with MUPP1 takes place between the PDZ binding motif of CAR and PDZ domain 13 of MUPP1.

Various proteins of the AJC including CAR are starting to be considered as tumor suppressors (for review see (102)). In the case of CAR, binding of its cytosolic domain to microtubules decreases the migration capacity of glioma cells (103), and CAR over expression in CAR deficient cell lines, primary cancer cells (104,105) and L-fibroblast (99) reduces cell proliferation. Interestingly this

capacity is lost upon deletion of the PDZ binding motif of CAR. Since ablation of this motif does not affect the junctional localization of CAR, it is proposed that CAR exerts its cell growth regulatory activity through its interaction with several PDZ containing proteins. In this respect it has been shown that besides ZO-1 and MUPP-1, CAR interacts with the following PDZ proteins: a) MAGI-1b, an inverted MAGUK protein present at the TJ of epithelial cells. MAGI-1b is considered a tumor suppressor since the tumorigenic potential of the viral oncoproteins E4-ORF1 of the human adenovirus type 9 and E6 of the high risk human papillomavirus depends on the viral ability to sequester or to target MAGI-1 for degradation (106); b) Protein interacting with protein C kinase (PICK1). This molecule is a PKC phosphorylation substrate that serves as an adaptor between transmembrane receptors and PKC; c) Ligand-of-Numb proteins X1 and X2 (LNX1 and LNX2). These proteins have multiple PDZ domains and interact through their second PDZ repeat with the C terminus tail of CAR (107,108). Numb is a phosphotyrosine-binding (PTB) domain-containing protein, regulator of Notch, a protein implicated in asymmetric cell division (109). LNX proteins cause proteosome-dependent degradation of Numb and enhance Notch signaling (110,111). Recently it has been observed that the carboxyl terminus of JAM-4 binds LNX1 second PDZ domain and that LNX1 facilitates the endocytosis of JAM4 (112) and d) PSD-95/SAP-90, a MAGUK protein present in neuronal synapsis. This interaction might be relevant during development, since CAR is strongly expressed in the mouse neuroepithelium of the neural tube, the developing brain and the spinal cord, while its expression decreases postnatally and is absent in adult tissues (113).

Another example where CAR expression changes dramatically with development is the heart. This is particularly interesting since adenoviruses and coxsackie viruses are the most important causes of viral myocarditis. In rat CAR is highly expressed on fetal and newborn but not adult myocardium (114,115), and in human autopsy specimens CAR is expressed in the hearts of human fetuses but not in the hearts of young children and adults (116). Instead a strong CAR signals is present at the intercalated discs and sarcolemma of patients with dilated cardiomyopathy (DCM) (117). These observations hence suggest that low CAR abundance may render normal human myocardium resistant to CAR-dependent viruses, whereas re-expression of CAR, such as that observed in DCM, may be a key determinant of cardiac susceptibility to viral infections. With regards to the intercalated discs that connect myocardial cells end to end, it is pertinent to mention that in them other proteins of the AJC like ZO-1 and vinculin have also been detected (118,119,120).

CAR also interacts with integral proteins of the AJC. Thus a) in mammalian male germ cells CAR forms a complex with JAM-C. CAR appears already in early round spermatids, is confined to the acrosome and is present in mature spermatozoa. Interestingly CAR is exposed on the surface of acrosome reacted, but not acrosome intact cells (121); b) CAR is the epithelial counter receptor for JAML, a junctional adhesion molecule-like protein expressed in granulocytes that lacks the PDZ binding motif characteristic of JAM proteins (122). Upon bacterial invasions polymorphonuclear leukocytes (PMN) constitute the first line of host defense. This requires PMN to migrate out of the circulation, through the interstitium across the epithelium to face the offending pathogens. Migration of PMN along the lateral membrane of epithelial cells requires the key adhesive interaction of leukocyte $beta_2$ integrin CD11b/CD18 and its ligand JAM-C located at epithelial desmosomes (52,48), while migration across epithelial TJs involves the interaction of PMN expressed JAML with CAR present at the TJ of epithelial cells (123); and c) CAR co-immunoprecipitates with beta-catenin, although it is not yet known through which domains this interaction takes place (124).

Finally it should be mentioned that CAR binds to microtubules (103) and actin (125), suggesting a role of CAR in processes that involve the dynamic reorganization of the cytoskeleton.

4.1.2. Coxsackie virus

Coxsackie are enteroviruses of the *Picornaviridae* family. Their name was given after the town of Coxsackie on the Hudson River, where the first specimens of the virus were obtained (126). Coxsackie viruses are single stranded positive sense RNA viruses and the viral particle is a 30 nm icosahedron. The virus is distributed via the fecal-oral route and infection occurs after eating contaminated food (127).

Based on their pathology coxsackie viruses have been divided into: 1) group A that produces a common childhood illness known as hand, foot and mouth disease characterized by fever and painful blisters in the mouth, palms and fingers of the hand or on the soles of the feet (128); and 2) group B that induce fever, headache, sore throat, GI distress as well as chest and muscle pain (Bornholm disease) that can progress to myocarditis or pericarditis which can result in permanent heart damage or death (129). Coxsackie B infection may also induce aseptic meningitis and the B4 strain has been discovered to cause Diabetes mellitus type 1.

CAR is the receptor of group B coxsackieviruses (CVBs). However due to its localization at the TJ, CAR is inaccessible to virus approaching the epithelium from the apical surface. This situation is overcome by the initial interaction of CVBs with another cellular receptor, the decay accelerating factor (DAF) (130,131). This protein due to its glycosylphosphatidylinositol (GPI)-anchor displays an apical surface localization in polarized epithelial cells (132). Interaction of CVBs with DAF results in DAF clustering in lipid raft domains sensitive to cholesterol depletion, and activation of Abl (133). The latter is a non-receptor tyrosine kinase that mediates actin remodeling through Rac. This reorganization of actin facilitates the movement of the virus in the epithelial surface to the TJ region, allowing its subsequent interaction with CAR. Binding of CVBs to CAR promotes junctional disassembly reflected by a decrease in TER and triggers conformational changes in the virus capsid (formation of A

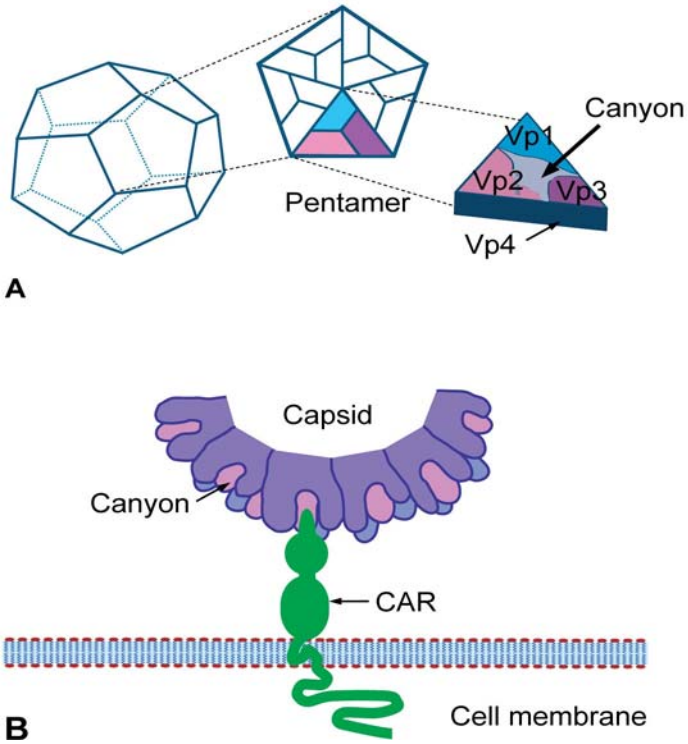

Figure 3. Schematic representation of the coxsackie virus canyon and its interaction with CAR. A) Coxsackie virus capsid is integrated by viral proteins VP1, VP2, VP3 and VP4. A narrow depression termed canyon found around each of the pentamer axes that constitute the capsid is the CAR binding site. B) CAR inserts into the canyon present on the capsid of coxsackie virus.

particles) that are essential for the posterior release of the viral RNA into the cytoplasm. Interaction of CVBs with DAF also activates the Src family kinase Fyn that phosphorylates caveolin-1. Viruses are finally internalized in vesicular structures that contain phosphorylated caveolin-1 but not clathrin markers. However since this process is not inhibited by dominant negative forms of dynamin II, a GTPase involved in budding of both clathrin coated (134) and caveolin containing vesicles (135,136), it has been concluded that CVB internalization route is a novel one that employs caveolae and a dynamin independent mechanism, sensitive to cholesterol depletion.

The coxsackie virus capsid is integrated by 60 copies each of four viral proteins VP1, VP2, VP3 and VP4 which form an icosahedral shell that encapsidates the RNA genome. A prominent feature of the capsid surface of picornaviruses is a narrow depression termed canyon found around each of the pentamer axes that constitute the capsid (137) (Figure 3). The canyon has been predicted to be the receptor binding site and it has been suggested to be protected from immune surveillance by being less accessible to relatively large antibodies. The external proteins VP1, VP2 and VP3 participate in receptor binding, but the majority of the interactions with CAR are with VP1 (138). Cryo-electron microscopy has been employed to analyze the three dimensional structure of coxsackie virus B3 (CVB3) in complex with full length human CAR and also with the the first and second Ig-like domain fragment of CAR (138), revealing that: 1) Pairs of transmembrane domains of CAR associated with each other bind to CVB3 capsids with higher avidity than the monovalents first Ig-like domain and first and second Ig-like domains fragments. 2) The first Ig-like domain of CAR binds into the canyon of CVB3. 3) The second Ig-like domain of CAR makes an angle of around 125^{o} with the long axis of the first Ig-like domain. 4) The hinge between the second Ig-like domain and the transmembrane domain is more flexible or longer than the hinge between the first and secong Ig-like domains.

Several residues in the coxsackie virus capsid line the binding interface with CAR: E1089, W1090, V1091, P1146, G1147, G1148, V1150, S1197, E1198, S1200, N1202, G1203, V1204, N1208, N1211, N1212, G1214, T1215, K1259, T1279, T2136, N2138, N2139, P2141, S2164, N2165, K2166, Y3091, S3181, N3182, E3183 (residues in VP1, VP2 and VP3 are numbered starting with 1000, 2000 and 3000 respectively) (138). These residues are well conserved among the six coxsackie virus serotypes as well as in swine vesicular disease virus (SVDV), another virus that also uses CAR as its cellular receptor (see below). The mutation N2165 in VP2 attenuates the myocarditic phenotype and might influence the electrostatic interaction between the virus and E28 or E29 of CAR (139). The amino acids of human CAR identified in the CVB3 interface are almost completely conserved in mouse and include I24, T26, E28, E29, M30, K44, F45, L47, S48, N51, Q52, Q53 P54, N98, N99, K101,

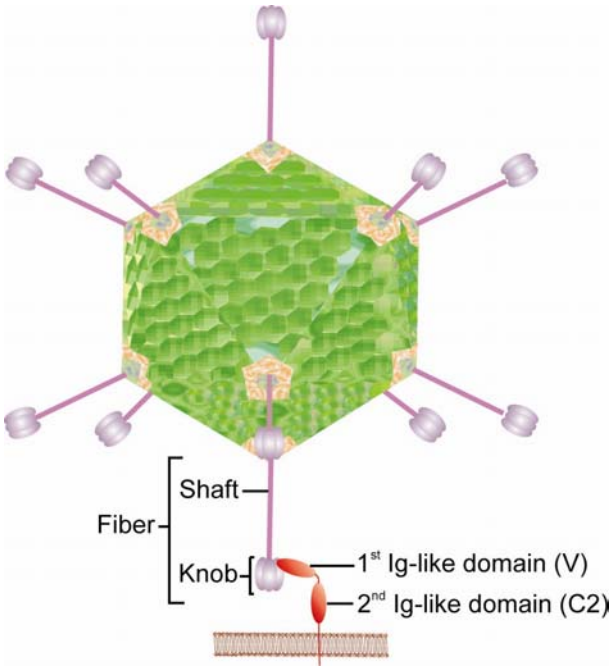

Figure 4. Schematic representation of the adenovirus fiber protein and its interaction with CAR. Fiber trimers project from each penton complex (orange) of the icosahedral capsid of adenovirus. Fiber trimers are integrated by a central shaft and a globular knob. The first Ig-like domain of CAR associates to the knob of the fiber protein that project from the adenovirus capsid.

S102, K125, K126, A127, P128, G129, V130, A131, K133, K134 and H136 (138).

4.1.3. Swine vesicular disease virus

SVDV belongs to the *Picornaviridae* family and the genus *Enterovirus*. SVDV produces a relatively new pig disease whose first outbreak occurred in Italy in 1966 (140) and later appeared in Hong Kong, Japan and a series of European countries. SVD is characterized by fever and vesicles with subsequent ulcers in the mouth, snout, feet and teats. Animals exhibit lameness and an unsteady gait, shivering and jerking type leg movements. The disease is transmitted by eating infected meat, by contact with infected animals or infected feces.

CAR is the cellular receptor for SVDV, as CHO cells expressing human CAR, but not CHO mock transfected controls are susceptible to SVDV infection, produce progeny SVDV and develop cytopathic effects (141). Additionally, SVDV infection can specifically be blocked with a monoclonal antibody against CAR, and with antibodies against the decay accelerating factor CD55, like occurs with several strains of coxsackie virus group B.

Interest has developed in studying the relationship between this pig enterovirus and Coxsackie B virus. Thus it has been shown that Coxackie virus B5 antiserum has the ability to neutralize SVDV, and that pigs exposed to Coxackie B5 virus develop SVDV neutralizing antibodies (142). These results have raised the speculative proposal that the virus might have been transferred from man to swine.

4.1.4. Adenovirus

Adenoviruses are small, non-enveloped icosahedral viruses containing double stranded DNA of the family *Adenoviridae*. Their name derives from the adenoids (tonsils) where they were initially isolated. Adenoviruses have been classified into 6 distinct groups A to F on the basis of their genetic variability, oncogenic potential and G + C content of their DNA (143). They have been identified as causative agents of different diseases with clinical manifestations in gastrointestinal (F primarily and A), respiratory (B, C and E) and ocular (D and E) epithelia. Adenoviruses are transmitted by coughed-out droplets, contact with an infected person as well as by fecal routes (143).

CAR is the cellular attachment protein for adenovirus serotypes from subgroups A, C, D and F (144). The exception to CAR binding is provided by the subgroup B (144) and some subgroup D serotypes that cause epidemic keratoconjunctivitis, for whom CD46, a complement regulatory molecule functions as a receptor (145,146).

The mechanism of infection of adenoviruses is mediated by a protein complex called the penton located at the vertices of the viral capsid. The penton is composed of an elongated fiber protein and a base protein (Figure 4). The latter facilitates adenovirus entry by binding to alpha$_v$ integrins through the integrin binding sequences RGD and LDV (147,148). Adenovirus binding to the first Ig-like domain of CAR is mediated by its fiber protein, and more specifically by its knob domain (87). Adenovirus fiber

protein can disrupt CAR homodimer formation, as it has a higher affinity for CAR binding than CAR itself (149,150).

In many cells including the thoroughly studied model of HeLa, the interaction of the knob region of fiber with CAR is responsible for the initial attachment of the adenovirus to the plasma membrane of the target cell, whereas the association of the penton base with the cell surface integrins is responsible of triggering virus uptake by clathrin mediated receptor endocytosis (151,152). Upon internalization the adenovirus is delivered to endosomal compartments where it undergoes lysis, escapes to the cytosol (153) and then interacts with microtubules and dynein/dynactin for its transit to the nucleus. The penton base appears to be involved in this process (154,155). Viral entry is cholesterol dependent (156), but not due to viral internalization through caveolae (157). Instead recent evidence suggests that CAR is associated with GM1 ganglioside containing lipid raft microdomains (158), thus explaining why disruption of these regions by cholesterol depleting agents (e.g. methyl-beta-cyclodextrin) inhibits adenovirus infection (156). All together these results indicate that binding of adenovirus fiber protein to CAR followed by penton protein binding to $alpha_v$ integrins triggers a cholesterol dependent clathrin mediated endocytosis of the virus.

Interestingly, a somewhat different mode of adenovirus infection is present when instead of using cancerous cells like HeLa (human cervical adenocarcinoma) or A549 (human pulmonary carcinoma), secretory epithelial cells of the lacrimal gland (LGAC) are employed (157). In this model the penton base capsid protein of adenovirus 5 remains surface associated, while the knob domain of the fiber capsid is rapidly internalized. Fiber but not penton base protein stimulates macropinocytosis, a process that is important for efficient adenovirus 5 intracellular trafficking, but that does not participate directly in adenovirus 5 entry. Hepatocytes which lack expression of $alpha_v beta_3$ and $alpha_v beta_5$ integrins constitute another cell type that has been reported to utilize a penton base independent internalization mechanism for adenovirus 5 (159,160).

In principle it was not easy to understand how could an apically present adenovirus employ as cell receptors proteins like CAR and integrins that are respectively located at the TJ and the basolateral membrane? And in fact the early attempts to treat cystic fibrosis by gene therapy employing non replicating adenoviral vectors were a failure since basolateral localization of fiber receptors limited adenovirus infection from the apical surface of airway epithelia, obstructing the virus capacity to deliver the therapeutic genes in sufficient quantities (161,162). It is proposed that wild type adenoviruses access CAR and their basolateral integrin receptors by transient breaks in epithelial integrity, and that this does not constitute a problem since wild type infection requires only a few epithelial cells to be infected as subsequent replication expands viral numbers (163). Interestingly in a polarized epithelial airway model newly replicated adenovirus particles are released only to the basolateral surface (124). Viruses subsequently cross to the apical surface through the paracellular pathway. The adenovirus fiber protein that is produced during viral replication facilitates the apical escape, as binding of fiber to CAR disrupt TJ integrity, observed by a drop in TER. The appearance of the adenoviruses on the apical surface can then allow the spread of the infection to a new host or to a different part of the lung.

4.2. Reovirus and feline calicivirus utilize JAM-A as their cellular receptor

4.2.1. JAM-A

The human JAM-A gene is composed of 13 exons encoding two groups of mRNA that differ in length and sequence (164). Type 1 cDNAs are shorter at the 5' end and contain a region not found in type 2. Type I messengers contain exons E1-E10 whereas type 2 messengers contain exons E1a, 1c part of E1 and E2-E10. Two alternative promoters regulate the expression of these messengers. Type 1 mRNAs are present in endothelial cells, platelets, white blood cells and in several epithelial cell lines, while type 2 messengers are limited to endothelial cells.

hJAM-A is a protein of 299 amino acids, that exhibits 215 residues in the extracellular domain and 45 amino acids in the cytoplasmic region (165) (Figure 2A). Both Ig loops are of V type. Due to N-glycosylation at the membrane proximal Ig loop at residues N185 and N191, this protein gives rise by SDS-PAGE to a band of 36-41 kDa instead of the predicted one of 30 kDa. The N-terminal Ig loop is crucial for the formation of U shaped JAM-A homodimers oriented in cis on the cell surface that interact in trans with JAM dimers located at an opposite cell surface (166,49). In this respect it is pertinent to mention that the structural homology between sigma1 and fiber, the reovirus and adenovirus attachment protein respectively (Figure 6), also extends to their respective receptors, which form similar dimeric structures (167).

JAM-A contributes to the gate function of TJs. Thus JAM-A antibodies and a soluble extracellular fragment of it fused to the IgG Fc domain, inhibit the recovery of TER in a Ca^{2+}-switch assay (165), and the over-expression of JAM-A, diminishes the permeability of paracellular tracers in epithelial monolayers (55).

JAM-A is expressed on the surface of dendritic cells, platelets and leukocytes and at the TJ of epithelial and endothelial cells including those responsible for the blood-brain barrier (168,55,169,40).

4.2.2. Reovirus

Reovirus are non enveloped icosahedral particles with ten segments of double stranded RNA encapsidated within two protein shells, the inner core and the outer capsid. The name reovirus is due to their original isolation from the respiratory and enteric tracts of human and animals and to the observation that no disease could initially be associated to them, a situation that earned them the name of orphan viruses. The present nomenclature includes in the viral family *Reoviridae* viruses of several

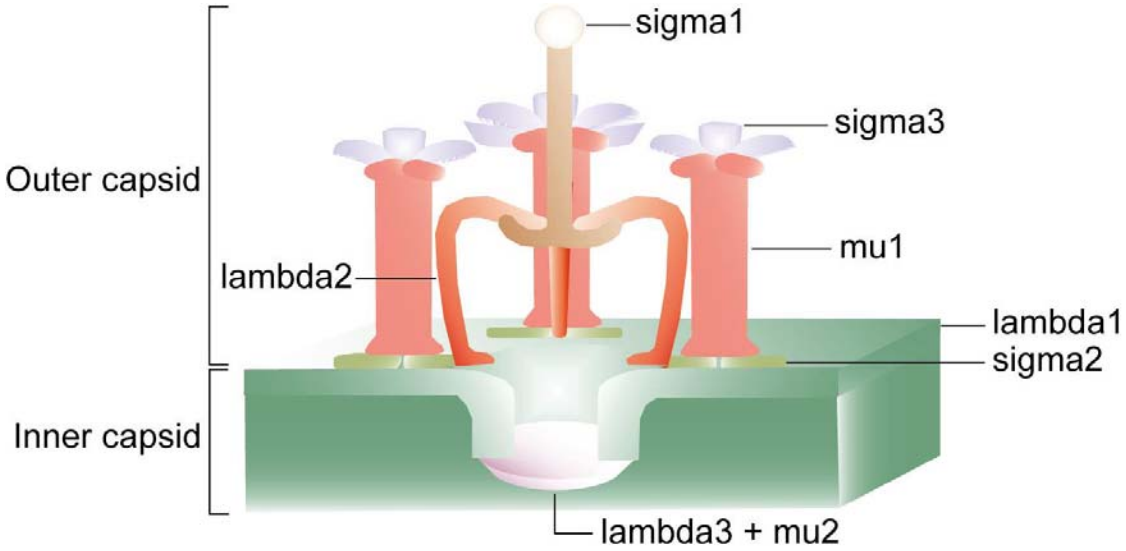

Figure 5. Molecular organization of the outer capsid of the reovirus virion. Schematic cross-sectional view of the virion capsid. Observe the location of the sigma3 protein that is removed in the endosomal compartment allowing the exposure of protein mu1. Note how protein sigma1, the molecule that interact with JAM-A, has a head that projects from the virion surface.

genera, which are causative agents of disease (e.g. rotaviruses), while the original reoviruses have been incorporated into the genus *Orthoreovirus (*170). The avian orthoreoviruses are important disease agents; instead orthoreovirus infections of mammals are usually asymptomatic and are therefore considered as generally benign (171). An exception is found with neonatal mice, which are exquisitely susceptible to reovirus infection (172,173). In these animals type 1 (T1) reovirus strain spreads hematogenously from the intestine to the central nervous system (CNS) and shows tropism for ependymal cells, while type 3 (T3) reoviruses spread via neural routes to the nervous system where they infect neurons. T1 strain causes non lethal hydrocephalus, whereas T3 strain causes lethal encephalitis. Additionally myocarditis has become a well established experimental model of reovirus induced disease in mice (174).

In humans, reovirus infection is benign and the immense majority of the population becomes infected early in life and has specific serum antibodies by early adulthood. These viruses enter the host by either respiratory or enteric routes and infect the epithelium and associated lymphoid tissue (175). After attachment of reovirus to its target cells surface receptors, the virus is internalized via receptor mediated endocytosis in clathrin vesicles. In the endo/lysosomal compartment acidic pH and the action of several proteases allow the removal of the major outer capsid protein sigma3, resulting in the exposure of viral outer capsid protein mu1 (Figure 5). The latter is a N-terminal myristoylated protein that penetrates into the membrane of the endosome allowing the exit of the transcriptionally active viral core into the cytoplasm, where replication proceeds (for reviews on reovirus entry see (176,177).

JAM-A was identified as the receptor of reovirus by transfecting the reovirus resistant cells COS-7 (green monkey kidney fibroblasts), with a cDNA library derived from reovirus susceptible human neuronal precursor cells NT2. The cells that had acquired the ability to bind strain 3 reoviruses expressed a cDNA that encoded JAM-A (57). The ability of JAM-A to function as a reovirus receptor was further confirmed with antibodies against JAM-A that blocked binding of reoviruses to a variety of target cells, and by plasmon resonance studies which demonstrated that a fusion protein comprising the extracellular domain of JAM-A could bind to the head domain of reovirus sigma1 protein (Figure 5). Moreover, the use of a monoclonal antibody specific for a conformational epitope within the sigma1 head domain blocked binding of reovirus strain 3 to soluble JAM-A. Since strain 3 reoviruses interact with sialic acid on the epithelial surface, and alpha-sialyllactose, a competitive inhibitor of reovirus binding to sialic acid could not inhibit the association of sigma1 head domain to soluble JAM-A, it was concluded that reovirus binding to JAM-A occurs via the virion head and does not require sialic acid. Binding of sigma1 to reovirus is of high affinity with a calculated K_d of 6 x 10^{-8} M. Further studies demonstrated that JAM-A also serves as a receptor for strain 1 and 2 reoviruses whereas the related JAM family members JAM-B and JAM–C do not function as receptors to any type of reovirus strains (178).

By employing a structure guided mutational analysis of JAM-A, the reovirus binding determinants were identified. Hence it was demonstrated that sigma1 binds to JAM-A monomers and that residues E61 and K63 of beta strand C and L72 of beta strand C' of JAM-A are required for the efficient engagement to strain 3 reoviruses (179). These residues are located in the first Ig-like domain of

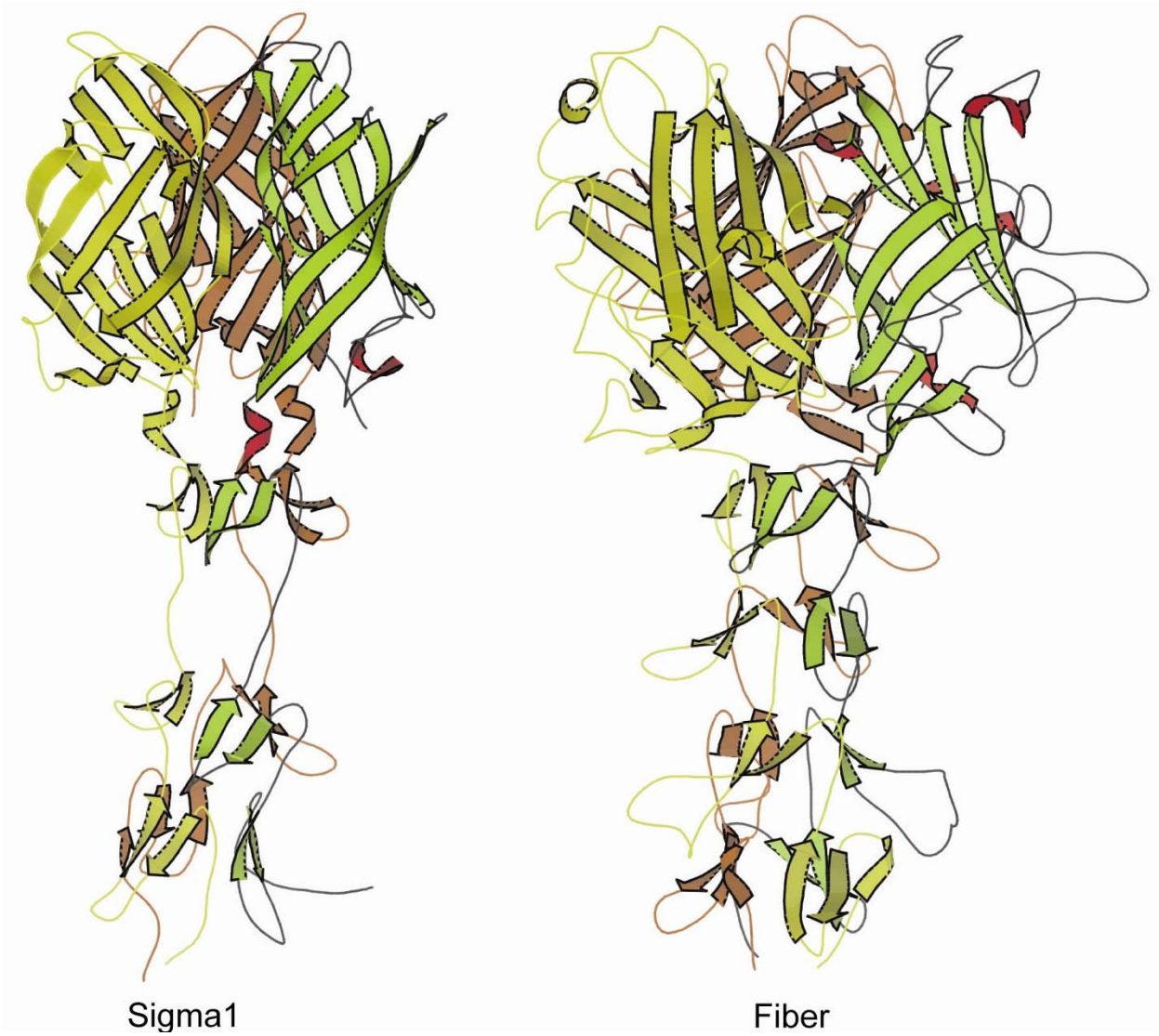

Figure 6. Comparison of the overall structural topology of reovirus sigma1 protein and fiber protein from adenovirus. Both viral proteins sigma1 and fiber have a trimeric structure that exhibits a head and tail morphology with a triple beta spiral forming the tail and an eight stranded globular domain integrating the head. sigma1 protein from reovirus binds to cellular JAM-A, whereas adenovirus fiber associates to CAR. Both JAM-A and CAR are JAM proteins of the immunoglobulin superfamily and are present at the TJ of epithelial cells. This figure was prepared using KiNG program and taking the information provided in (339,181)

JAM-A, which is required for the homodimerization of the molecule.

Reovirus sigma1 protein is an elongated trimer with a head and tail morphology. The N-terminal tail of sigma1 partially inserts into the virion via turrets formed by the pentameric lambda2 protein present in the outer viral capsid, while the C-terminal sigma1 head projects from the virion surface (180) (Figure 5). Crystal analysis of sigma1 reveals that the head domains contain three beta-barrel domains, one for each trimer, constructed by eight antiparallel beta strands (181). Sequence analysis and structural modeling suggest that the N-terminal half of the tail is formed from an alpha-helical coiled coil (182). Interestingly the overall structural topology of sigma1 is remarkable similar to that of fiber (181,183) (Figure 6), the adenovirus attachment protein, that associates to CAR another protein of the JAM family present at the TJ of epithelial cells. Since reoviruses and adenoviruses belong to different virus families and have few properties in common, the observed similarities between sigma1 and fiber suggest a conserved mechanism of attachment and a probable ancient evolutionary relationship.

The similarity between reovirus sigma1 protein and adenovirus fiber protein has prompted the development of a new gene delivery vehicle in which the potent adenovirus 5 vector is genetically reengineered to display sigma1 protein of reovirus type 3 in order to make it capable of targeting cells expressing JAM-1 and sialic acid

instead of CAR (184). This chimeric vector could in principle allow the transduction of dendritic cells which are deficient in CAR (185) but do express JAM-1 (186). Dendritic cells are considered the most powerful antigen presenting cells and therefore constitute an important target for vaccine vectors.

4.2.3. Feline calicivirus

Feline caliciviruses (FCVs) are non enveloped, positive sense and single stranded RNA viruses (187) that belong to the genus *Vesivirus* and the family *Caliciviridae*, named after the latin word *calyx*, that reflects the cup-shaped form of these viruses (188). Studies on virus of the family *Caliciviridae* have been limited because of the lack of cell culture methods for most of them. In contrast FCV replicates easily in cell culture and therefore has been thoroughly studied even after considering that it does not infect human tissues.

FCV causes respiratory infection in cats and other species of the Felidae family such as cheetahs (189,190). Symptoms in infected cats include fever, rhinitis, pneumonia and oral ulcers and in some animals polyarthritis, which may develop acutely, chronically or not at all (191,192). Although morbidity is high, mortality is usually low except in kittens as a consequence of pneumonia. A form of FCV called virulent systemic feline calicivirus (VS-FCV) causes a severe systemic disease by direct invasion of epithelium and endothelium and secondary host immune response, that in addition to ulceration and nasal or ocular discharge produces profound fever, anorexia, subcutaneous edema of the limbs and face, and alopecia (193).

FCV infects cells via clathrin mediated endocytosis and not by caveolae (194). The clathrin coated vesicles containing the virus then deliver their content into endosomes. Endosomal acidification is crucial for viral infection as it is required for uncoating the viral genome and allowing its subsequent access to the cytoplasm. Employing a cDNA expression library derived from feline kidney cells, JAM-A was identified as the cellular binding molecule of FCV (195). Additionally it was demonstrated that the expression of feline or simian JAM-A into the non permissive human embryonic kidney epithelial cells 293T, allows binding and infection by numerous FCV strains. Instead human JAM-A can be recognized by FCV but cannot support virus infectivity in transfected cells. Although no clear explanation to this situation is yet available, it has been observed that feline and simian JAM-A have two amino acid residues conserved in the extracellular domains (S91 and K155) that are not preserved in human JAM-A, which might play a major role in FCV infectivity (Figure 2A).

4.3. Alpha herpes virus 1 (HSV-1) and 2 (HSV-2), pseudorabies virus, bovine herpes virus 1 and poliovirus associate with nectins and nectin-like proteins

The name herpes viruses derives from the Greek word *herpein*, meaning to creep, since a notable characteristic of the viruses of the *Herpesviridae* family is to remain as persistent or latent infections for the lifetime of the host and to become reactivated when it becomes stressed or immunocompromised (196).

Herpes viruses have been classified into three subfamilies on the basis of biological characteristics and genomic analysis (197). Members of the alphaviruses subfamily are neurotropic, have a short replicative cycle and display a broad host range. Human alpha herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1) are representative members of the alphaherpes virus subfamily and will be here described since they employ as cellular receptors the AJC proteins nectins 1 and 2, and necl-5.

4.3.1. Nectins 1 and 2 and nectin-like 5

Nectin-1 mediates the entry of HSV-1, HSV-2, PRV, and BHV-1 (198), whereas nectin-2 is the receptor of HSV-2 and PRV (199). The amino terminal V like domain of nectins 1 and 2 is necessary and sufficient for binding the gD protein of alpha herpes viruses. Directed mutagenesis and hybrid forms of nectins 1 an 2 have revealed that loops between the C' and C'' beta strands and between the F and G beta strands of the V like domain are critical for gD binding (200,201,202).

Defects in humans and knockout mice have highlighted the tissues where nectin molecules play an essential function that is not readily compensated by related proteins. Thus human homozygosity for a nonsense mutation of nectin-1 (W185X) results in an autosomal recessive disease named cleft lip with or without cleft palate ectodermal dysplasia (CLPED1)/Zlotogora-Ogür syndrome, characterized by cleft lip/palate, syndactyly, mental retardation and ectodermal dysplasia, frequently observed in northern Venezuela (Margarita Island) (203,204). Nectin-1 knock out mice suffer microphtalmia, skin abnormalities and abnormal mossy fiber trajectories in the hippocampus (205,206), whereas nectin-2 knock out mice have male specific infertility (207).

Necl-5 also known as Tage4/poliovirus receptor/CD155, mediates the entry of PRV, BHV-1 and poliovirus (198,208). Ncl-5 has four splice variants of which alpha and delta are membrane bound and have a short cytoplasmic domain. Ncl-5 isoforms beta and gamma arise from alternative splicing of exon 6 encoding the transmembrane domain of the molecule (208). In normal human tissues from adult autopsies, Western blot analysis shows expression of ncl-5 in renal cortex, ileum, liver, spinal cord, brain stem, cerebellar cortex and motor cortex tissues (209).

Necl-5 has no homophilic cell-cell adhesion activity but exhibits a Ca^{2+} independent trans heterophilic binding with nectin-3, involved in contact inhibition (210,211). Thus in moving cells, necl-5 localizes at the leading edge of cells where it forms in cis binary and/or ternary complexes with integrin $alpha_v beta_3$ and the platelet derived growth factor (PDGF) receptor (212,213). These complexes transduce signals involving Rap1 and Rac for

cell movement and Ras for cell proliferation. When these moving cells contact each other, they initiate the formation of cell junctions by establishing a Necl-5/nectin-3 trans interaction that induces activation of Cdc42 and Rac and the subsequent reorganization of the actin cytoskeleton (214). Additionally the downregulation of necl-5 by clathrin dependent endocytosis is generated (215). This leads to reduction in cell movement and proliferation. Nectin-3 dissociated from necl-5 is retained on the cell surface, and subsequently interacts with nectin-1 triggering E-cadherin recruitment and the eventual establishment of AJs.

Necl-5 interacts with vitronectin (216), a component of the extracellular matrix that binds to integrin $alpha_v beta_3$ and $alpha_v beta_5$ (217), thus suggesting its involvement in mediating cell to extracellular matrix adhesion. The cytoplasmic domain of ncl-5 alpha associates to the mu1B subunit of the clathrin adaptor complex AP-1B. This complex is required for the correct targeting of many basolateral proteins in epithelial cells such as the transferring receptor and the low density lipoprotein receptor (218,219). The association of ncl-5alpha to mu1B appears to be critical for sorting ncl-5alpha to the basolateral membrane of polarized epithelial cells, as ncl-5delta that lacks de mu1B subunit binding motif displays a non polarized distribution in the surface of epithelial cells (220). The cytoplasmic domain of ncl-5alpha and delta interacts with Tctex-1 (221), a light chain subunit of the dynein motor complex that represents the major driving force of retrograde transport of membrane bound organelles and vesicles and that is involved in spindle movement during mitosis. Both ncl-5 and Tctex-1 are expressed in neurons throughout the spinal cord giving a punctuate distribution pattern that suggests their localization in vesicular structures in the cell body or on the cell surface of neuronal synapses (222).

Necl-5 is also a recognition molecule for the natural killer (NK) cells. It interacts with CD226 and CD96 on NK cells to stimulate their cytotoxic activity (75,223). This condition might be critical for NK cell recognition of tumor cells, as ncl-5 is highly expressed in them.

4.3.2. HSV-1 and HSV-2

Human herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) belong to the subfamily *Alphaherpesvirinae* and the genus *Simplexvirus*. HSV-2 causes genital herpes, which is a sexually transmitted disease. HSV-1 produces the commonly called cold sores or fever blisters that occur on or near the lips. Additionally this virus can infect the cornea of the eye causing keratitis. These viruses have the capacity to enter sensory and autonomic neurons whose axons extend to the place of the lesions, and set up a latent infection (224). From time to time when the infection is reactivated the virions are transported from the neuron to the initial site of infection to cause recurrent lesions. HSV-1 reactivates more efficiently from trigeminal ganglia to cause cold sores or keratitis and HSV-2 reactivates more efficiently from lumbosacral dorsal root ganglia to cause genital herpes. However it should be mentioned that there are increasing number of cases where HSV-1 infects the genitals and HSV-2 infects the face.

Herpes viruses are organized in three structures: capsid, tegument and envelope. The viral genome is a linear double stranded DNA molecule housed in an icosahedral capsid surrounded by tegument. The viral envelope contains a large number of spikes composed of 10 or more glycoproteins, whose name is prefixed “g”.

Since Herpes are enveloped viruses, their entry to cells occurs as the envelope fuses with the plasma membrane, although infection may also occur by endocytosis followed by fusion between the virion envelope and the endosomal membrane. The release of intracellular Ca^{2+} is critical for the entry of HSV (225). Thus the interaction between the viral envelope and heparan sulfate glysosaminoglycan chains of syndecan-2 and nectin-1 triggers an increase in Ca^{2+} just bellow the plasma membrane, referred to as membrane Ca^{2+}. This Ca^{2+} mobilization although important is insufficient for viral entry and a further release of Ca^{2+} stores from the endoplasmic reticulum triggered by the interaction of the virus with integrin $alpha_v$ is required to complete the entry process. Once the nucleocapsid and the tegument proteins are released into the cytoplasm, the former is transported along microtubules to the nucleus for viral replication to take place, whereas tegument proteins are transported to several sites in the cell where they down regulate host DNA, RNA and protein synthesis (224,196).

Attachment of HSV-1 to host cells involves first the interaction of viral envelope proteins gB or gC with heparan sulphate and then of the gD HSV-1 glycoprotein with nectins in epithelial cells or to HVEM, a TNF family member in activated T lymphocytes. X-ray structures of HSV-1 gD ectodomain, crystallized either alone or in a complex with a portion of HVEM has revealed that the gD ectodomain is an Ig fold with unconventional disulphide bonding (226). An amino terminal extension from the Ig like fold forms a hairpin loop that contains the critical amino acids (7-15 and 23-32) involved in the interaction of gD with HVEM, nectin-2 and all the other entry receptors (e.g. 3-O sulfated heparin sulfate in the case of HSV-1) except for nectin 1 (227). Apparently the interface in gD that interacts with nectin-1 lies downstream of amino acid 32 and outside of the N-terminal hairpin.

Polarized epithelial cells have been employed in order to study for which epithelial surface the apical or the basolateral, the Herpes simplex viruses display a preferred tropism. Kidney epithelial cells MDCK cultured on tissue culture inserts proved not to be susceptible to apical HSV-1 infection unless TJs were opened by depletion of extracellular Ca^{2+}. In contrast these cells became infected when HSV-1 was added to the basolateral surface (228). These results hence suggested that the cell receptor for the virus was located on the lateral membrane bellow the TJ seal. In a similar fashion confluent cultures of MDCK cells and human keratinoyctes became infected only after wounding the cell monolayers, and infected cells were observed next to the wound where basolateral membranes were accessible. Moreover, in subconfluent cultures, infected cells were detectable in the peripheral cells of islets (229). Taken together these results support virus

penetration via basolateral membranes. However the opposite observation has been made when working with different human cell lines. Thus when human uterine (ECC-1), colonic (Caco-2) and retinal pigment (ARPE-19) epithelial cells were grown on collagen coated inserts, they were 16 to 50 fold more susceptible to HSV-1 and HSV-2 infection at the apical surface than at the basolateral surface (230). Disruption of TJs by Ca^{2+} chelation with EGTA overcame the restriction on basolateral infection and had no impact on apical infection. However it is interesting to observe that in this work nectin-1 appears by confocal imaging to be distributed along the apical surface of Caco-2 cells instead of being concentrated at the uppermost portion of the lateral membrane where the AJC is located, and where nectin-1 has been found to localize upon transfection in MDCK cells (231). All together these results suggest that HSV tropism depends on the membrane surface where nectin-1 is expressed in each particular cell type.

The preference of HSV to infect the lips, the genitals and the eye, and to remain latent in the nervous system is certainly associated to the elevated expression of nectin-1 in these tissues. For example: 1) In the murine eye nectin-1 is widely expressed among cells of the corneal epithelium and endothelium, conjuntiva, lens epithelium, ciliary body, iris, choroids and retina (232). 2) Nectin-1 transcripts have been detected in the adult mice by *in situ* hybridization in neurons in sensory, sympathetic, and parasympathetic ganglia of the peripheral nervous system, in neurons of the ventral and dorsal horns of the spinal cord and of the brain stem, cerebellum, cerebral cortex, hippocampus, dentate gyrus and olfactory bulb (233). 3) Human homozygotes for a nonsense mutation of nectin-1 (CLEPD1) exhibit cleft lip, revealing the importance of this molecule in lip development and interestingly human CLEPD1 heterozygotes are resistant to infection by alpha-herpesviruses (203). 4) In the human vaginal epithelium nectin-1 is highly expressed throughout the menstrual cycle and instead in the mouse vaginal epithelium nectin-1 is expressed only during the diestrus, the stage of the estrus cycle most permissive for HSV infection (234).

Herpes viruses have developed a way of contiguous cell-cell spread that involves the formation of intercellular bridges through which virus can pass from an infected to a virus naive cell (235). This system allows the virus to escape from extracellular detrimental molecules such as antibodies, complement and enzymes as well as from elimination from phagocytes. This process of virus spreading requires the participation of mu1beta, a subunit of the clathrin adaptor complex AP-1B involved in targeting basolateral proteins, and of HSV proteins gE/gI, since mutant HSV virions lacking gE or its cytoplasmic domain do not reach the cell junctions and instead are found on the apical surface or accumulated in the cytoplasm (236). Interestingly the interaction of nectin-1 with afadin, an actin filament binding proteins, increases the efficiency of HSV-1 cell spread (237). This situation may be due to the fact that in the cell-cell adhesion system at cadherin based AJs, nectin is associated with cadherin through the interaction between afadin and alpha-catenin (238).

4.3.3. Pseudorabies virus

Pseudorabies in the nineteenth century was a disease linked to CNS disorders in cattle, dogs and cats, characterized by itching, rubbing, exhaustion and paralysis. Accordingly it was called “mad-itch” disease (239). Later the Hungarian veterinarian Aladar Aujeszky demonstrated the infectious origin of the disease and revealed that it was reminiscent but distinguishable from rabies, thus naming it pseudorabies or Aujeszky’s disease (240).

The pseudorabies virus (PRV) that belongs to the family of the *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Varicellovirus* infects a broad range of vertebrates including pig, cattle, sheep, dogs, cats chickens, rodents, rabbits and some non human primates like rhesus monkeys and marmosets. The virus can be transmitted via nose to nose or fecal-oral contact, while indirect transmission occurs via inhalation of aerosolized virus.

The dramatic increase in swine production and close-quarter confinement of large number of pigs in barns in the last fifty years has provided the ideal condition for the spread of PRV. In pigs the virus primarily replicates in the respiratory tract, especially in the nasal and oropharyngeal mucosa (241), and then spreads along cranial nerves to the brain and via lymph and blood to internal organs, being the reproductive organs important targets. Younger swine are the most severely affected by PRV infection and typically exhibit symptoms of central nervous infection while older pigs exhibit symptoms of respiratory disease (242). It is pertinent to note that although the stratum corneum and the epidermis of the porcine snout are thicker than those of human lips, the porcine snout has been proposed to function as an equivalent *in vitro* model for human lips, as they display similar morphology and histology (243). Therefore it might be speculatively proposed that the infection of swines through the snout and nasal mucosa is due to an elevated expression in these tissues of nectin-1 as appears to be the case for human lips.

PRV virion consists of a double stranded DNA genome surrounded by a capsid and an envelope that consists of a bilayered phospholipid membrane. The space between the capsid and the envelope is filled with tegument proteins. Attachment of the virus to its target cells is initiated by binding of the viral envelope glycoprotein gC to the cellular heparin sulfate proteoglycans exposed at the plasma membrane. Next PRV bounds via its glycoprotein gD with three cellular receptors nectin-1, nectin-2 and necl-5 (244,197). Binding is followed by fusion of the virus to the cellular plasma membrane and the transportation of the viral capsids to the nucleus via microtubules (245). Porcine nectin-1 has 96% amino acid identity to human nectin-1 and upon transfection to Chinese hamster ovary CHO K1 cells that do not express any of the known alpha herpes virus receptors, makes the cells susceptible to entry of HSV-1, HSV-2, PRV and bovine herpes virus (246). PRV gD protein binds to porcine nectin-1 with a 10 fold higher affinity than HSV-1, a fact that may help to explain the particular tropism of these viruses. The importance of nectin-1 for PRV infection is further highlighted in

experiments where transgenic mice are made resistant to PRV infection by expressing soluble forms of porcine nectin-1 (247,248).

4.3.4. Bovine Herpesvirus-1

Bovine herpesvirus-1 (BHV-1) is an alpha herpes virus of the genus *Varicellovirus* that causes severe health problems in young calves and newborn animals that include rhinotracheitis, conjunctivitis, genital infections (e.g. vulvovaginitis and balanoposthitis) and an upper respiratory infection referred to as "shipping fever" (249). BHV-1 is not the sole agent that contributes to the latter disease, as other virus like bovine viral diarrhea virus, bovine parainfluenza-3 and bovine respiratory syncytial virus are also involved. BHV-1 is a critical factor in the development of shipping fever since by immunosuppressing the infected animals (250,251,252,253), the cattle is left predisposed to opportunistic secondary bacterial infections that can cause pneumonia. In adult animals BHV-1 causes only mild clinical signs or reduce milk production, although reduced fertility and abortions have also been observed. BHV-1 remains in a latent state in the trigeminal ganglia of the host animal, and its reactivation is induced by stress triggered by inclement weather, transportation or overcrowding of animals (254,255). BHV-1 can spread through aerosol transmission, sexual contact, frozen semen and embryos.

The entry of BHV-1 into cells requires the interaction of viral gD glycoprotein with nectin-1 or necl-5 (198). The binding sites for BHV-1 gDs on nectin-1 overlap but are not identical to those present in the gDs of HSV-1, HSV-2 and PRV. For example, it has been observed that while residues Q76, N77 and M85 present at the N-terminal variable immunoglobulin domain of nectin-1 are critical for entry of HSV-1 and HSV-2, they are not important for the entry of BHV-1 or PRV (202).

4.3.5. Poliovirus

Poliovirus (PV), the causative agent of poliomyelitis is a member of the *Picornaviridae* family of the genus *Enterovirus*. The viral genome is constituted by a single stranded sense RNA molecule enclosed in a nonenveloped capsid comprising 60 copies of four different polypeptides arranged with icosahedral symmetry.

Human and non-human primates are the only natural host for PV. By transforming mouse L fibroblasts with human (HeLa) cell DNA, the previously resistant fibroblasts became susceptible to PV infection (256). This observation eventually led to the isolation of the poliovirus receptor cDNA, and the encoded receptor turned out to be ncl-5/CD155 (257).

The amino terminal variable Ig like domain of ncl-5 interacts with PV viral protein 1 (VP1) in the canyon that surrounds each of the twelve five fold axes of the icosahedral viral capsid (258,259,260). However due to the orientation of the long and slender ncl-5 molecule relative to the PV surface, the interaction is akin to a "sausage in a bun" (261). Two theories have been postulated for PV cell entry. In the first one following receptor docking, the N-terminal of VP1 and the myristoylated viral protein 4 (VP4) are extruded from inside the virion and inserted into the cell membrane (262). This leads to the formation of a 135S viral particle also known as A particle (263) and of pores in the membrane through which the virion RNA could be injected into the cytoplasm (264). This model proposes that the empty capsid is left behind at the cell surface and does not enter the cell. However it remains a matter of controversy whether poliovirus entry requires an obligatory transition to the 135S particle (265). In the second model PV is taken up in endosomes (266) in a process independent of dynamin, thus excluding the participation of clathrin vesicles or caveoli for virus uptake (267).

PV as a typical enterovirus replicates very efficiently in the GI tract particularly in the oropharyngeal and intestinal mucosa (268). PV receptor ncl-5 has been detected in the germinal centers of tonsils and Peyer's patches as well as their follicle associated epithelium and on enterocytes on the small intestine and colon (269,216). Viruses shed in the feces of the infected persons are responsible for transmission of the disease. From the GI tract viruses drain into cervical and mesenteric lymph nodes and then to the blood causing a transient viremia. At this stage the infection only causes a minor disease characterized by sore throat, fever and malaise. Replication at extraneural sites that include brown fat, reticuloendothelial tissues and muscle, is believed to maintain viremia beyond the first stage and to increase the possibilities of entry into the CNS. This latter stage only occurs in 1-2% of infected individuals and leads to viral replication in motor neurons within the spinal cord, brain stem or motor cortex. Viral replication in these neurons provokes their destruction and leads to muscle paralysis and, in severe cases to respiratory arrest and death.

Not all tissues that express ncl-5, such as the liver and kidney are sites of poliovirus replication (209,208,257). An explanation for this situation in liver could rely on the fact that this organ mainly expresses ncl-5 soluble isoforms beta and gamma that may compete for receptor binding sites on the virion. Other possibility relies on the fact that tissue tropism is not solely governed by the availability of the appropriate viral receptors, but also on whether the virus finds a cell internal environment suitable for replication. By this we refer to the presence of host factors involved in the initiation of translation, proteolytic processing of the viral polyprotein, RNA replication, etc.

Wild type mice are resistant to infection with poliovirus due to the fact that they do not express ncl-5. However when ncl-5 transgenic animals were engineered they were not susceptible to oral infection with PV, and could only be infected by intracerebral, intravenous or intramuscular route (270,271). Analysis of these mice revealed that they express very low levels of ncl-5 in the intestine and that none was present in Peyer's patches (269), thus indicating that the presence of ncl-5 in these locations is crucial for oral susceptibility to PV infection. In order to ascertain whether necl-5 is the sole determinant of PV susceptibility in the mouse intestine, another transgenic mice was generated by using the promoter for

rat intestine fatty acid binding protein to direct necl-5 expression in the mouse gut. Interestingly although ncl-5 was detected in the enterocytes and M cells of the small intestine of this transgenic animal, upon oral inoculation no viral replication was detected in the small intestine and no increase in virus titer was found in the feces. These results strongly suggested the participation of other factors in determining the ability of PV to replicate (272). One of such factors appears to be the production of alpha/beta interferon (IFN). Thus PV infection of ncl-5 transgenic mice that lack the receptor for alpha/beta interferon resulted in viral replication in liver, spleen and pancreas, in addition to the CNS (273). Ncl-5 is present in all these tissues, but PV only replicates in the brain and spinal cord of ncl-5 transgenic mice that synthesize the alpha/beta interferon receptor. Thus it was concluded that the alpha/beta interferon receptor protects the extraneural organs from PV infection. Since replication at extraneural sites appears to be required for virus entry into the CNS it is speculated that in 98-99% of PV infections the IFN alpha/beta response prevents invasion of the CNS (274).

Two theories prevail to explain how PV enters the CNS. In the first the virus is proposed to pass from the blood to the CNS by crossing the blood-brain barrier (275). This process is independent of the presence of ncl-5, as the viruses are delivered to the CNS in the same amount and rate in ncl-5 transgenic and non transgenic mice. The mechanism employed by the virus to cross the BBB remains unknown, but is rather effective as the virus accumulation in the CNS is more than 100 times higher than that of albumin and only three times lower than that of monoclonal antibody against transferrin receptor. The second hypothesis proposes that the virus is transported by retrograde axonal transport ascending from the muscle to the spinal cord and brain. The observation that ncl-5 interacts with Tctex-1 a component of dynein retrograde motor complex, supports this proposal (221). Both hypotheses depend on the presence of the virus in the blood, and in this respect it has been demonstrated that PV can replicate in primary human monocytes (276,277).

PV uptake at the presynaptic axon terminal is believed to happen in a different manner to the uptake observed in tissue cultured cells, as the viruses appears not to uncoat immediately and to be transported in the form of intact 160S particles (278). This condition is favorable for the virus since the naked RNA genome would unlikely survives the transit to the cell body where genome replication takes place.

4.4. Hepatitis C virus associates to claudins -1, -6 and -9.

4.4.1. Claudin-1

Claudins -1 and -2 were the first members of the claudin family identified as molecular constituents of TJs (279). Claudin-1 is present in a wide variety of epithelia. Thus in the kidney it is found in Bowman's capsule (280) and the distal and collecting tubules that correspond to the tighter segments of the mammalian nephron (67,280). In the inner ear claudin-1 is present at the cochlea, the organ of Corti, the marginal cells of the stria vascularis, Reissner's membrane, spiral limbus, and the sensory epithelium and dark cells of the vestibule (281). Claudin-1 is also present in the lung (282,283), liver (284,285), ovary (286), prostate (287), uterine epithelium (288,289) and Sertoli cells of the testis (290,291). In mouse testis claudin-1 expression increases with development and accordingly testosterone treatment significantly augments claudin-1 expression in immature Sertoli cells (290). In neonatal mice claudin-1 is localized in the dorsal surface of the tongue and glandular compartment of the stomach but is absent from the oral mucosa and the keratinized compartment of the stomach (292). It is also present in the differentiated and/or undifferentiated compartments of the epidermis.

In human epidermis claudin-1 is expressed in the whole epithelium, in contrast to other TJ proteins such as occludin, ZO-1 and claudin-4 whose expression is restricted to the granular layer (293). The presence of claudin-1 at the epidermis appears to be crucial since knock out mice die within one day of birth due to acute dehydration (294). In these animals a subcutaneously injected paracellular tracer reaches the skin surface revealing that claudin-1 exerts a crucial role for the maintenance of the epidermal barrier.

The expression of claudin-1 is altered in transformed tissue. Thus in poorly differentiated hepatocellular carcinoma the expression of claudin-1 is attenuated and in those cases that show portal invasion the diminished expression is even more significant (295). In human breast tumors and breast cancer cell lines claudin-1 expression is low or undetectable (296,297). Surprisingly when the genomic organization of claudin-1 was assessed in hereditary and sporadic breast cancer as well as in breast cancer cell lines, no genetic alterations in the promoter or coding sequences of claudin-1 could be identified that could explain the loss of claudin-1 expression, hence suggesting that other regulatory or epigenetic factors may be involved in the down-regulation of this gene during breast cancer development (298). Interestingly when claudin-1 is re-expressed in claudin-1 negative human breast tumor cells (MDA-MB-361), the gate function of TJs is restored as the paracellular flux of dextran decreases (299). Maintaining a certain level of expression of claudin-1 appears to be important to avoid epithelial transformation. Hence over-expressing claudin-1 also appears to exert a deleterious effect. For example, in malignant epidermal disorders like squamous cell carcinoma and Bowen's disease claudin-1 is strongly expressed in tumor cells with keratinization such as cancer pearls as well as in unkeratinized tumor cells (293). The over-expression of claudin-1 has also been observed in human esophagus squamous cell carcinoma (SQCC) (300), gastric intestinal type adenocarcinomas (301), and in colorectal cancer (302,303). Interestingly nude mice injected subcutaneously with human colon cancer cells (SW-480) that over-express claudin-1 ($SW480^{claudin\text{-}1}$), develop more and bigger size tumors than those animals injected with untransfected SW-480 cells ($SW480^{control}$). Moreover, the animals injected with $SW480^{claudin\text{-}1}$ cells had a 100% incidence of liver metastasis while those treated with $SW480^{control}$ had none (303). It is pertinent to mention that claudin-1 over-expression in cancerous

epithelia, does not indicate the existence of better sealed TJs. In fact the opposite appears to be true. Thus in colorectal cancer despite claudin-1 over-expression the tissue is permeable to paracellular tracers like ruthenium red and displays a significant disorganization of TJs strands observed in freeze-fracture replicas (302). These observations hence suggest that claudin-1 over-expression could have a potential utility as a diagnostic biomarker and could possibly constitute a target for therapeutic intervention as has proven to be the case for the novel cancer therapy that relies on treating carcinomas that over-express claudin-3 and -4 with *Clostridium perfringens* enterotoxin (CPE) as this toxin elicits specific cytolysis of claudin-3 and -4 expressing cells (304,305,306,307).

4.4.2. Claudin-6

The sequence similarity of certain mouse EST clones with claudins -1 and -2 allowed the identification of a new member of the claudin protein family that was subsequently named claudin-6. Since Northern blot studies were unable to detect claudin-6 expression in adult mouse tissues, and the EST sequences that correspond to claudin-6 were from embryos, it was concluded that the expression of claudin-6 could be developmentally regulated (308). This early prediction proved to be true as further studies have revealed that claudin-6 has a high level of expression in neonatal mice proximal tubules, thick ascending limb of Henle's, distal convoluted tubules and collecting ducts, while no expression is detected in the adult kidney (309). Moreover, in the mouse submandibular gland, claudin-6 expression is restricted to the ducts at embryonic days E14 and E16, but after birth remains undetectable (310). Remarkably in mouse embryonic stem (ES) cells differentiating into embryoid bodies, claudin-6 is one of the earliest molecules to be expressed in the cells committed to the epithelial fate, and the onset of its expression coincides with the expression of the early epithelial marker keratin 8, thus suggesting that claudin-6 might be involved in the early epithelialization of the mouse embryo (311). In neonatal mice claudin-6 is present in the differentiating suprabasal compartment of the epidermis, nail and oral mucosa and in the dorsal and ventral surface of the tongue and the keratinized squamous epithelium of the stomach (292). Additionally, in the columnar epithelium of the glandular stomach claudin-6 reveals a non membranous pattern of expression.

Transgenic mice that over-express claudin-6 via de involucrin promoter, exhibit an increased transepidermal water loss that results in lethality within 24 to 48 hours of life (312). Barrier disfunction further evidenced by the penetration of X-gal through the skin is accompanied by a decreased expression of claudins -3, -4, -7, -8, -10, -11 and -14, and abnormalities in the expression of epidermal differentiation markers that participate in the formation of the insoluble cornified envelope (CE) including the over expression of keratin 1, filaggrin, involucrin, loricrin, transglutaminase 3 and small proline rich proteins (SPRRs) 2D and 2G and the down regulation of keratin 6/16, repetin, SPRR1A and 2A, and the Kruppel-like transcription factor 4 (Klf4) involved in the regulation of SPRR2A. These results suggest that the expression of claudin-6 in the subrabasal layer of the epidermis regulates both the structure and function of this epithelium. It is noteworthy that the epidermal phenotype of these transgenic mice is reminiscent of the skin of premature human infants, characterized by a deficient epidermal barrier responsible for the occurrence of poor temperature stability, infections by micro-organisms through the skin and water outflow.

Interestingly in the organ of Corti at the inner ear claudins -6 and -9 are detected in a novel hybrid intercellular junction, which has been designated "tight-adherens junction" (TAJ) (313). This TAJ is a large 3-5 μm junction that constitutes the only point of contact between the mechano-sensory outer hair cells (OHCs) and their supporting Deiter cells (DCs) in the organ of Corti. The most apical region of the TAJ is formed by parallel TJ strands rich in claudin-14 and an underlying scaffold of ZO-1 that assembles a small actin cytoskeletal network. A second subdomain located bellow is enriched in claudins -6 and -9 and forms an extensive and anastomosing network of TJ fibrils that has an underlying ZO-1 scaffold enriched with the canonical adherens junction proteins p120, alpha- and beta-catenins that recruit an extensive actin cytoskeletal network

4.4.3. Claudin-9

In the kidney claudin-9 appears to be developmentally regulated (309). Thus in adult mouse kidney claudin-9 is non detectable, while in 1 day old proximal convoluted tubules its mRNA can be detected by real time PCR. Western blot analysis further revealed the presence of claudin-9 in one day old kidneys, its diminished expression at two weeks of age and its absence in adult animals. Transcripts for claudin-9 have also been identified in human corneal epithelium (314). In mice inner ear claudin-9 has been detected at the cochlea in the organ of Corti, at the stria vascularis, in the Reissner's membrane and spiral limbus, in the vestibule and in the dark cell area (281).

4.4.4. Hepatitis C virus

Hepatitis C virus (HCV) is a 50 nm, enveloped, single stranded, positive sense RNA virus of the genus *Hepacivirus* of the family *Flaviviridae*. This virus is distinct both genetically and clinically to hepatitis A and B viruses. After infection HCV causes liver inflammation that is often asymptomatic. Approximately 20-30% of persons clear HCV from their bodies during the first 6 months after infection, but the remaining 70-80% of patients infected develop chronic hepatitis C that can result later in cirrhosis and liver cancer.

HVC is spread by blood to blood contact with an infected person's blood. Around 170 million people are infected with this virus around the world.

The lack of a robust system allowing the production of HCV in cell culture delayed for many years the study of HCV infection. Recently two major advances have surmounted this condition. First, infectious retroviral particles pseudotyped with HCV envelope proteins (HCVpp) have been generated to study virus receptor

usage. Second, a cell culture model has been developed that enables the study of virus attachment, internalization and fusion. Such model consists of hepatoma cells transfected with the full length HCV genome derived of a HCV strain isolated from a patient with fulminant hepatitis. Transfected cells produce HCV particles (HCVcc) that can infect naive hepatoma cells. Employing these novel systems it was demonstrated that HCV enters the hepatic cell by clathrin mediated endocytosis (315). HCV infection is sensitive to agents that interfere with the acidic pH of endosomes, a situation explained by the fact that clathrin coated vesicles deliver their content into endosomes with an acidic content. Low pH is proposed to trigger the fusion of the viral envelope with the membrane of the endosome leading to the delivery of the viral genome into the cytosol.

The HCV genome encodes three structural proteins, capsid protein C and envelope glycoproteins E1 and E2 that form a heterodimer (316). Diverse cellular proteins have been shown to interact with E2 including: 1) CD81, a cell-adhesion protein present in the basolateral surface of epithelial cells. This tetraspanin protein with two extracellular loops of different lengths interacts with E2 via its large extracellular loop (317). 2) The scavenger receptor B type I (SR-BI) (318). This multiligand receptor primarily expressed in the liver and steroidogenic tissues, binds a variety of lipoproteins including high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL). It has been suggested that HCV interacts with SR-B1 through associated lipoproteins and not directly through E2. 3) The LDL receptor (319). Mammalian cells take up LDL particles in order to get the phospholipids and cholesterol needed to build new membranes. LDL binds to LDL receptor located on the plasma membrane and is internalized by clathrin mediated endocytosis. HCV present in infected sera associates with LDL and VLDL. It is thought that HCV piggybacks on lipoproteins as they enter the cells through their interaction with the LDL receptor. 4) The lectins L-SIGN (320) and DC-SIGN (321). These proteins are not expressed in hepatocytes. L-SIGN is present on endothelial cells of liver sinusoids, whereas DC-SIGN is present on dendritic cells. Therefore it is proposed that as liver endothelial cells and the dendritic cells of the liver (Kupffer cells) are located adjacent to hepatocytes they may capture and transfer HCV to hepatocytes. 5) The asialoglycoprotein receptor (ASGPR) has been found to bind recombinant E1 and E2 proteins produced in insect cells (322). ASPGR is a protein found in liver cells that clears from the circulation, by receptor mediated endocytosis, glycoproteins that lack terminal sialic acid residues. Since insect cells cannot attach sialic acid residues to glycoproteins, the binding of ASPGR to E1 and E2 produced in insects' cells has been questioned (323). 6) The heparin sulfate proteoglycans (324). This association that provides a primary docking site for the virus is proposed to proceed by the interaction of the positively charged amino acid residues that are highly conserved in the N terminus of E2 with the negatively charged residues of the highly sulfated heparin proteoglycans found on the cell surface.

Claudin-1 has recently been identified as a HCV co-receptor (325). This evidence was obtained employing a cyclic lentivirus cDNA library repacking screen, in which a complementary DNA library derived from a highly HCV permissive hepatocarcinoma cell line was packed and applied to a $CD8^+$ $SRB1^+$ non permissive human kidney epithelial cell line (293T) which was then challenged with HCVpp. As a result of this screen claudin-1 was identified as the factor that specifically rendered 293T cells susceptible to HCVpp. Neither claudin-7, the closest relative of claudin-1 (60% amino acid identity) nor claudin-3, a protein expressed in liver with a high identity to claudin-1 (49% identity) rendered 293T cells permissive to HCVpp. Claudin-1/claudin-7 chimeras constructed by exchanging in claudin-7 different segments of its extracellular loops for those present in claudin-1, were tested for their ability to allow transfected cells to become HCVpp susceptible. The amino terminal third of the first extracellular loop of claudin-1 was identified as crucial for HCV binding and further point mutation studies demonstrated that residues I32 and E48 located within this segment are critical for virus interaction (Figure 2B). Interestingly E48 is conserved in all the species where claudin-1 has been sequenced, whereas I32 is conserved in many species (humans, rat, mouse and rabbit) and in bull is substituted by V (Figure 7A). Furthermore, upon comparing the first extracellular loop of several human claudins we observe that I32 is only present in claudin-1 although it is substituted by V in claudins -3, -5, -6, -8, -9, -10, -11, -15, -17 and -20, and for L in claudin-23; whereas E48 is conserved in claudins -1, -3, -4, -6, -8, -9, -17 and -19 (Figure 7B).

The importance of claudin-1 for HCV infection is further highlighted by observing that even a moderate down regulation of claudin-1 with siRNA inhibits HCV infection of the highly HCV-permissive hepatocarcinoma Huh 7.5 cell line. This result might help to understand the particular tropism of the HCV virus, as it could explain why low levels of claudin-1 expression in extra hepatic tissues may be insufficient to support HCV entry. In this respect it should be mentioned that the seminal work of Tsukita's group that describes the discovery of claudins, namely 1 and 2, was done isolating junctional fractions from chicken liver and that in this work it is mentioned that although claudin-1 mRNA is present in all the mouse tissues examined that include heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis, it is only observed in large amounts in the liver and kidney (279).

Finding that the human hepatocellular carcinoma cell line Bel7402 is permissive for HCV, even when it does not express claudin-1, triggered the search of other claudins that could mediate HCV entry. Thus it was demonstrated that claudins -6 and -9 but not -2, -3, -4, -7, -11, -12, -15, -17 and -23 are able to mediate the entry of HCV into target cells (326). Interestingly claudins -6 and -9 are expressed in the liver where HCV replicates the most. Moreover, these claudins but not claudin-1, were shown to be expressed in peripheral blood mononuclear cells, an additional site of

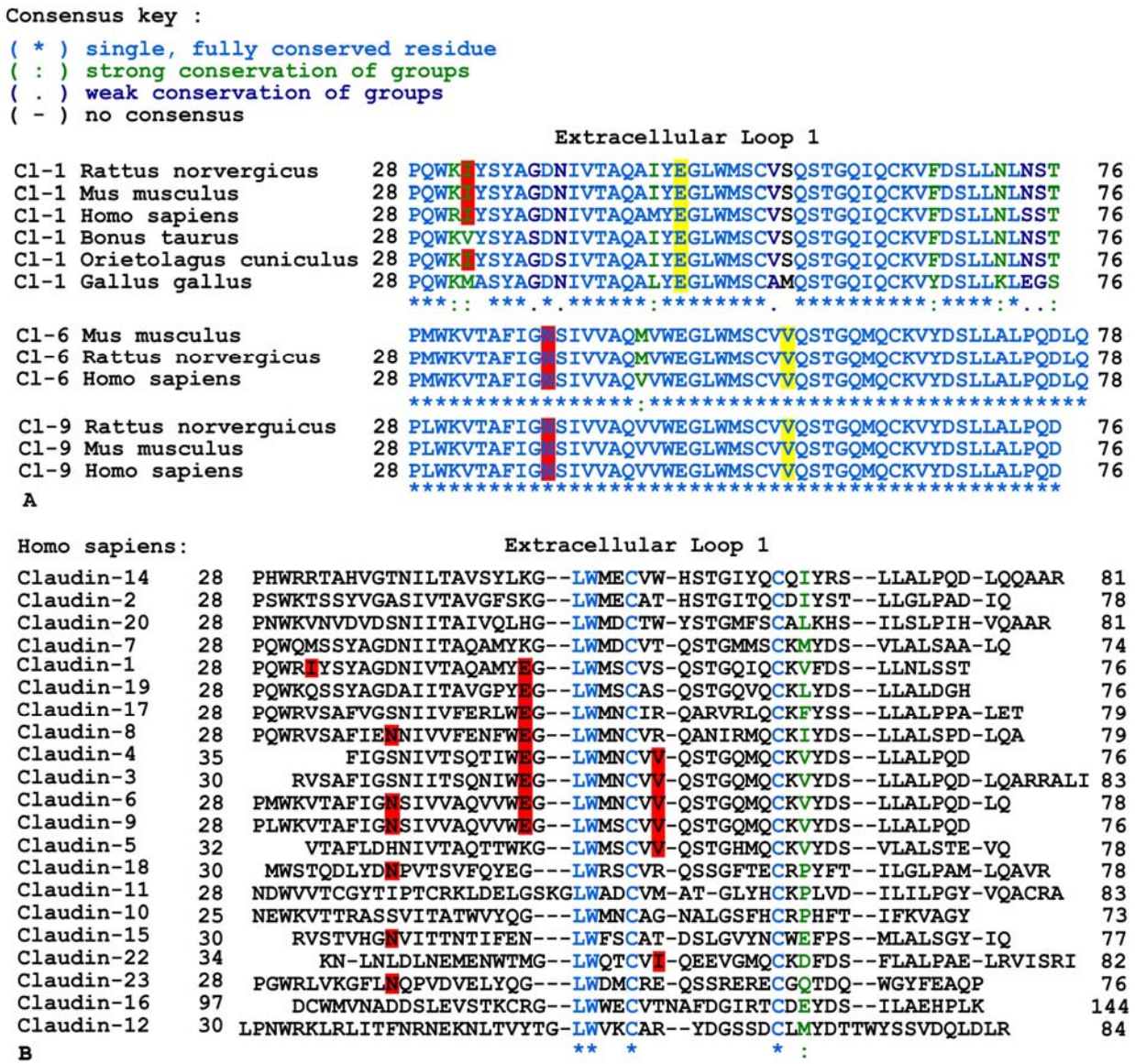

Figure 7. Comparison of residues critical for the interaction with HCV that are present in human claudins -1, -6 and -9 with those present in the sequence of other animal species and in other claudins. A) Comparison of I32 (red) and E48 (yellow) of the first extracellular loop of claudin-1 with residues present in this segment in other species (Upper). Comparison of N38 (red) and V45 (yellow) present in claudin-6 with residues present in this segment in other species (Middle). Comparison of N38 (red) and V45 (yellow) present in claudin-9 with residues present in this segment in other species (Lower). B) Comparison of the residues critical for the interaction with HSV found in claudins 1, -6 and -9 (red) with residues present in the first extracellular loop of different claudins from *Homo sapiens*.

HCV replication. Two residues N38 and V45 present in the first extracellular loop of claudins -6 and -9 were identified as critical for HCV entry. These sites are conserved in all the species where claudins -6 and -9 have been sequenced (Figure 7A). Furthermore, upon comparing the first extracellular loop of several human claudins we observe that N38 is preserved in claudins -6, -8, -9, -15, -18 and -23, while V45 is conserved in claudins -3, -4, -5, -6, -9 and -22 (with I) (Figure 7B).

A study in the human colon carcinoma cell line Caco-2 was performed with the aim of analyzing the effect of cell polarization on HVC entry (327). After several days in culture Caco-2 cells establish TJs with clear gate and fence properties that block the movement of tracers like dextran through the paracellular pathway and display a polarized distribution of proteins in the plasma membrane. In this condition the expression of HCV receptors CD81, SR-B1 and claudin-1 is increased and claudin-1 passes from having restricted location at the uppermost portion of the lateral membrane to being distributed along the whole basolateral surface. Surprisingly when HCVpp infection is

done in Transwell inserts either from the apical or basolateral surfaces, HCVpp entry is significantly higher via the apical surface. These results pose a conundrum since CD81, SR-B1 and claudin-1 display a polarized distribution in the basolateral membrane, and are therefore inaccessible to HCVpp due to the presence of TJs. Moroever, since it was observed that TJ opening by calcium depletion led to a 10 fold increase in HCVpp infection. These results could be explained if HCVpp infectivity is promoted in these cells by the interaction of the viral pseudoparticles with certain molecules present on the apical surface. The nature of such molecules remains to be defined. Additionally it can be speculatively postulated that HCV added to the apical surface may have the capacity to open the TJ and in such a way gain access to its cellular receptors located in the lateral membrane, in a manner similar to that exerted by rotavirus in epithelial cells (328).

Hepatocytes within the liver are highly polarized, possessing TJs that separate the basolateral (sinusoidal) from the apical (canalicular) domains. HCV is likely to enter the liver through the sinusoidal blood and hence to have initial access to the basolateral receptors in hepatocytes. In liver tissue claudin-1 is expressed in the apical and basolateral surfaces of hepatocytes, but interestingly only in the basolateral surface claudin-1 co-localizes with the HCV co-receptors CD-81 and SR-B1 (329). These results hence suggest that *in vivo* the basolateral surface of hepatocytes is more apt than the apical membrane to sustain HCV infectivity. Additionally it should be mentioned that HCV infection significantly increases claudin-1 expression levels in hepatoma cells.

4.5. Rotavirus

Rotaviruses are the leading cause of dehydrating diarrhea in infants and young children worldwide. These viruses of the Reoviridae family have a genome constituted by 11 double stranded RNA segments surrounded by three concentrical layers of proteins. From the outer layer formed by a protein named VP7, 60 spikes project outwards formed by a protein named VP4. The infectivity of rotaviruses depends on the specific proteolytic cleavage of VP4 into subunits VP5 and VP8. *In vivo* rotaviruses have a specific cell tropism, infecting primarily the mature enterocytes of the villi of the small intestine; while *in vitro* they can bind to a wide variety of cell lines including those of renal and intestinal origin.

Rotavirus cell entry is a complex multistep process (for review see (330)), in which proteins found within cell membrane lipid microdomains such as heat shock protein 70 (hsp70) and several integrins like $alpha_2beta_1$, $alpha_vbeta_3$, $alpha_xbeta_2$ and $alpha_4beta_1$ function as receptors for rotavirus. In accordance rotavirus surface proteins contain integrin ligand sequences. Thus the VP5 subunit of VP4 contains the $alpha_2beta_1$ integrin ligand site DGE, and VP7 the $alpha_xbeta_2$ and the $alpha_4beta_1$ integrin ligand sites GPR and LDV (20). This observation prompted us to ask how could a virus contained in the lumen of the intestinal cavity, employ as cellular receptors proteins like integrins that localize at the basolateral membrane below the TJ. In consequence we hypothesized that this could only happen if the virus had devised a way to open the TJ. Upon exploring the effect of the surface proteins of rotavirus on epithelial MDCK monolayers, we observed that rotavirus protein VP8 opens the TJ in a reversible and dose dependent manner (328).

Although the TJ proteins that interact with VP8 have not yet been identified, it is worth mentioning that upon analyzing the predicted amino acid sequence of VP8 we found that several segments of this protein have a high similarity to domains present in the extracellular loops of claudins and occludin (328). This observation posses the possibility that rotavirus VP8 proteins might be opening the TJ by competing with the homotypic interactions established among the extracellular domains of claudins and occludin, in a manner similar to that exerted upon the addition of peptides corresponding to the extracellular loops of occludin (331-335).

5. CONCLUDING REMARKS

Viral entry into epithelial cells is regulated by the interaction of viral proteins with numerous cell receptors. In this review we have described how proteins of the AJC located at the TJ and AJ as well as along the lateral membrane are employed by a wide array of viruses as cell receptors. Although many of these proteins are present in numerous epithelia, not all of them can sustain viral infection highlighting the fact that viral tropism does not require the presence of a single receptor but of sets of cell receptors that have to be expressed in a certain critical amount. Additionally, viral tropism depends on finding a cell internal environment suitable for replicating the viral genome. The capacity of certain viruses to infect is further limited by other factors such as the expression in the tissue of the alpha/beta interferon receptor.

The Ig superfamily is the most abundant family of cell surface molecules, and the analysis of the human genome reveals that Ig-like domains have the widest representation on any protein domain, being encoded in 765 genes (336). Ig-like domains are an evolutionary success story thought to be due to their stability, which is able to resist the harsh proteolytic and oxidative environment of the extracellular world (337). Therefore it is not surprising to find that so many viruses employ as cell receptors proteins containing such ubiquitous and resitant domains. The fact that so many proteins containing Ig-like domains are concentrated at the AJC gives viruses an additional advantage as it promotes junctional disassembly. This condition allows the viruses to breach the epithelial barrier and to gain access to the interior of the organism and to other cell receptors located on the basolateral surface of epithelial cells. It is also pertinent to mention that since junctional regions are sites of enhanced membrane recycling, endocytic uptake and intracellular signaling (338), by infecting through the AJC viruses might gain control of these important physiological functions.

It is noteworthy how both the viruses and host cells have developed sophisticated strategies to cope with

each other presence. Thus it is remarkable that some viral proteins disrupt the formation of homodimers of junctional proteins, by evolving a higher affinity for the receptor than that which the receptor has for itself. It is also outstanding how cells have generated an antiviral defense by producing soluble isoforms of AJC proteins that inhibit virus infection. This strategy could probably be employed in the future as a therapy against viral infection.

Last we should say that the identification of viral attachment receptors in epithelial cells has important implications for the future as it would help our understanding of virus tropism, pathogenicity and the mechanisms of virus entry. This knowledge will be crucial in order to improve gene therapy, medical therapy for virus infections and biological research.

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Abbreviations: AJ: adherens junction, AJC: apical junctional complex, ASGPR: asialoglycoprotein receptor, BHV-1: bovine herpesvirus 1, CD55: decay accelerating factor, CLPED1:cleft lip with or without cleft palate ectodermal dysplasia, CNS: central nervous system, CTX: cortical thymocyte Xenopus, CVBs: coxsackieviruses group B, CSF: cerebrospinal fluid, DAF: decay accelerating factor, DCs: Deiter cells, DCM: dilated cardiomyopathy, DRMs: detergent resistant membranes, ES: embryonic stem, FCV: feline calicivirus, GI: gastrointestinal GPI: glycosylphosphatidylinositol, HCV: Hepatitis C virus, HDL: high density lipoprotein, HIV: human immunodeficiency virus, HSV-1: alpha Herpes virus 1, HSV-2: alpha Herpes virus 2, IFN: interferon, Ig: immunoglobulin, JAM: junctional adhesion molecule, JAML: junctional adhesion molecule-like protein, LDL: low density lipoprotein, Necl: nectin-like, OHCs: outer hair cells, PMN: polymorphonuclear leukocytes, PRRs: poliovirus receptor related proteins, PRV: porcine pseudorabies virus, PV: poliovirus, SR-B1: scavenger receptor B type I, SVD: swine vesicular disease, SVDV: swine vesicular disease virus, TAJ: tight-adherens junction, TER: transepithelial electrical resistance, TJ: tight junction, VLDL: very low density lipoprotein, VP: viral protein, VS-FCV: virulent systemic feline calcivirus

Key Words: Tight Junctions, Adherens Junctions, Claudins, Nectin, Nectin-Like 5, CAR, JAM-A, Coxsackie, Swine Vesicular Disease Virus, Adenovirus, Reovirus, Feline Calcivirus, Herpes Virus, Pseudorabies, Bovine Herpes 1, Poliovirus, Hepatitis C, Rotavirus, Review

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