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Minireview

The *Polyomaviridae*: Contributions of virus structure to our understanding of virus receptors and infectious entry

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ABSTRACT

This review summarizes the field's major findings related to the characterization of polyomavirus structures and to the characterization of virus receptors and mechanisms of host cell invasion. The four members of the family that have received the most attention in this regard are the mouse polyomavirus (mPyV), the monkey polyomavirus SV40, and the two human polyomaviruses, JCV and BKV. The structures of both the mPyV and SV40 alone and in complex with receptor fragments have been solved to high resolution. The majority of polyomaviruses recognize terminal sialic acid in either an α2,3 linkage or an α2,6 linkage to the underlying galactose. Studies on virus structure, receptor utilization and mechanisms of entry have led to new insights into how these viruses interact in an active way with cells to ensure the nuclear delivery and expression of their genomes. Critical work on virus entry has led to the discovery of a pH neutral endocytic compartment that accepts cargo from caveolae and to novel roles for endoplasmic reticulum (ER) associated factors in virus uncoating and penetration of ER membranes. This review will summarize the major findings and compare and contrast the mechanisms used by these viruses to infect cells.

Introduction

The family *Polyomaviridae* are classified as group 1 viruses in the Baltimore classification scheme as they contain double stranded DNA genomes. There is a single genus designation within the virus family (Polyomavirus) and this genus contains 14 different species that infect at least 8 different mammalian species. In addition to containing dsDNA genomes all members of this virus family are non-enveloped. The members of the family typically display restricted species and cell type specificity for lytic infection but many can induce the transformation of cells that do not support lytic infection. Host cell and species-specific transcription and replication factors contribute significantly to the restricted specificity displayed by these viruses (Feigenbaum et al., 1987; Lynch and Frisque, 1991; Tada et al., 1989). Virus-receptor interactions are also known to contribute to cell type specificity and more importantly to pathogenesis in vivo (Caruso et al., 2007; Chen and Atwood, 2002; Dubensky et al., 1991; Freund et al., 1991a; Freund et al., 1991b; Fried et al., 1981). Like many other virus families the polyomaviruses use multiple and often distinct receptors and entry mechanisms to infect cells. At high enough multiplicities many of these viruses can infect cells using alternative often poorly described mechanisms. The structures of two major members of the family have been solved in complex with receptor fragments and this has given insight into how these viruses recognize host cells (Liddington et al., 1991; Neu et al., 2008; Stehle and Harrison, 1997; Stehle et al., 1994). A major area of current investigation by several labs is focused on understanding the intracellular trafficking of polyomaviruses in host cells and the mechanisms involved in virus uncoating and delivery of the dsDNA genomes to the nucleus. This paper will overview the field of virus structure and entry from its beginnings to the present. The focus will be on the mouse polyomavirus, on SV40, and on the two major human polyomaviruses, JCV and BKV.

Virus discovery

The mouse polyomavirus (mPyV) was first described by Ludwig Gross in 1953 in his studies on the transmission of mouse leukemias (Gross, 1953). He noticed that mice inoculated with cell free extracts from leukemias were capable of not only transmitting leukemia to naïve animals but that some of the animals also developed parotid tumors. In classic experiments he found that the "parotid" agent could be distinguished from the mouse leukemia virus by filtration. Filters with larger pore sizes allowed both viruses to pass but filters of smaller pore size selectively trapped the leukemia agent indicating

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that the parotid agent was smaller than the leukemia virus. In the mid to late 1950s Stewart and Eddy followed up on these observations and found that the "parotid" agent was capable of inducing many different types of tumors when inoculated into mice and renamed the virus "poly"oma from the Greek for many tumors (Eddy et al., 1958; Stewart et al., 1958).

SV40 was discovered as a contaminant of formalin inactivated polioviruses that were grown in Rhesus monkey kidney cells (Sweet and Hilleman, 1960). The subsequent characterization of this virus as a papovavirus capable of transforming cells in vitro and inducing tumors in experimental animals was a cause for great concern as several hundred thousand individuals were inoculated with the contaminated vaccine (Girardi et al., 1962). This launched several decades of intensive research on SV40. This work on SV40 led to the discovery of many important cellular processes described elsewhere in this issue of the journal.

JCV and BKV were both identified in 1971 from immunosuppressed patients. JCV was islolated in human fetal brain cultures following transfer from the brain of a patient with PML (Padgett et al., 1971). It was given the name JCV after the patient's initials. BKV was isolated in VERO cells (African green monkey kidney cells) from the urine of a renal transplant recipient (Gardner et al., 1971). It was also named after the initials of the patient from whom it was isolated.

Virus–host cell interactions

Mouse polyomavirus

The first studies of polyomavirus host-cell interactions followed closely behind their initial discovery and isolation in mammalian tissue culture. Shortly after virus cultivation a number of groups began examining the interaction of mPyV with host cells using both biochemical and electron microscopy (EM) techniques. Infection of cells by the mPyV was envisioned as a multi-step process that included virus adsorption, virus penetration, and virus uncoating. Morphological studies documented that whole virions were taken up into cells by phagocytosis as membrane bound single particles and membrane bound aggregates (Mattern et al., 1966; Mattern et al., 1967). Virus particles then appeared between the nuclear membrane but viral particles were not seen within the nucleus at these early times. This led to the conclusion that viral particles uncoat between the nuclear membranes and before they reach the nucleus (as we will see later this interpretation is largely correct with some minor modifications).

In the early 1970s it became apparent that polyomavirus preparations contained significant numbers of defective interfering particles. The ability to plaque purify virus preparations allowed for an enrichment of infectious virions and for their separation from empty capsids by density gradient centrifugation. Detailed analyses of these individual populations by EM indicated that empty capsids and light populations of virions (pseudovirions) were endocytosed by two different pathways (Bolen and Consigli, 1979; Mackay and Consigli, 1976). Virions and pseudovirions were predominantly endocytosed as single membrane-bound particles that trafficked intact to the nucleus. Empty capsids were endocytosed as aggregates into large vesicles that later appeared to fuse with lysosomes, apparently destined for degradation. A model was developed showing these two opposing endocytic routes in the cell (Fig. 1).

The fact that virions and pseudovirions behaved differently than empty capsids led Consigli and colleagues to carefully examine the viral particles for subtle differences in protein composition. Using radiolabeled preparations they identified six species of VP1 (A–F) by isolelectric focusing (Bolen et al., 1981). Only virions and pseudovirions were found to contain species E leading the group to hypothesize that species E was critical for delivering virions and pseudovirions to the nucleus. Antibodies raised against virions that were found to be neutralizing specifically recognized species D, E, and F supporting this hypothesis (Bolen et al., 1981). The group then went on to attempt isolation of cellular receptors for mPyV by preparing anti-idiotypic antibodies to VP1 (Marriott and Consigli, 1985; Marriott et al., 1987). These antibodies blocked infection and pulled down several proteins associated with cell surfaces. The identities of these proteins were never determined and none were found to act as receptors for the virus.

The mPyV and the two human polyomaviruses, JCV and BKV are known to require sialic acid for binding to host cells as "receptor destroying enzyme" or neuraminidase inhibits the viruses' ability to agglutinate red blood cells and to infect cells (Table 1). SV40 was thought not to attach to sialic acid as it did not agglutinate red blood cells and neuraminidase treatment of host cells did not reduce infectivity (Clayson and Compans, 1989). However, SV40 does attach to a sialic acid containing receptor, ganglioside GM1, with sialic acid being the major contact point, but the narrow specificity of SV40 for GM1 prevents it from binding to carbohydrates on red blood cells. In

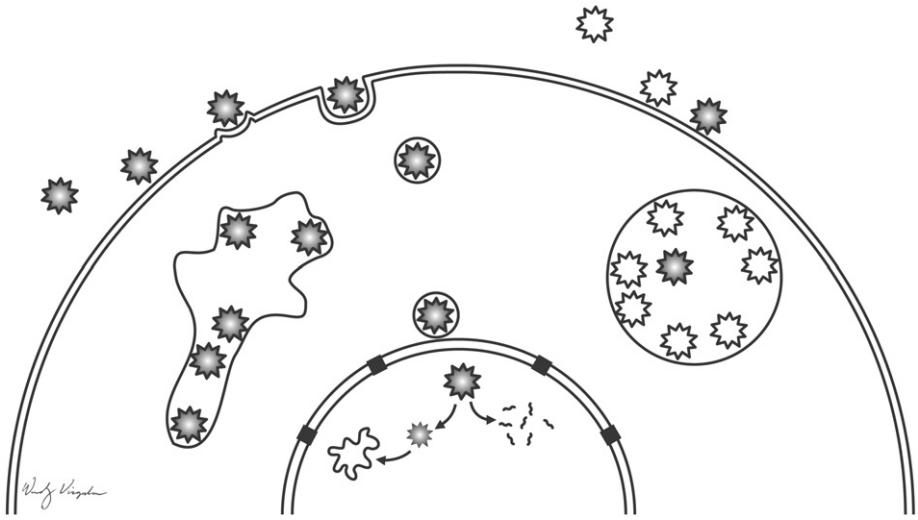

Fig. 1. Early model of polyomavirus attachment, penetration, and nuclear entry. Open virions represent empty capsids that are found to be internalized in large endosomal vesicles that presumably lead to degradation. Closed virions represent infectious particles and unlike empty capsids these are internalized into monopinocytotic vesicles. Some virions were found in large tubular structures that likely represent the pinching off of these structures from caveosomes described years later by Helenius (see text for detailed descriptions). Modified from information in Mackay and Consigli, 1976 and Maul et al., 1978.

Table 1
Receptors and entry pathways utilized by polyomaviruses

	BKV	JCV	SV40	mPyV
Receptors	α2,3-linked sialic acid	Terminal α2,3 or α(2,6)-linked sialic acid	Sialic acid on GM1	α2,3-linked sialic acid
	GD1b, GT1b	GT1b	GM1	GD1a, GT1b
Co-receptors	Unknown	Serotonin receptor (5HT-2a)	MHC class I	α4β1 integrin
Entry mechanism	Caveolae-mediated	Clathrin-dependent	Primarily caveolae-mediated	Caveolae dependent and independent mechanisms
Cytoplasmic transport	Vesicles localize to ER	Early endosomes to caveosomes	Fuse into caveosome and localize to ER	Endosome with caveolin-1 localizes to ER

ER, endoplasmic reticulum.

addition, the neuraminidase used for treating cells before challenge with SV40 does not cleave the sialic acid off GM1 so that it leaves the receptor intact (Miller-Podraza et al., 1982). It can even cleave sialic acid off higher order gangliosides to yield GM1. Therefore, neuraminidase treatment did not reduce SV40 receptors on cells and did not abolish infectivity. Early reports in the mPyV system suggested that the sialic acid interactions were largely due to non-specific electrostatic interactions between the virus and these negatively charged cell surface carbohydrates. Subsequent studies by the Paulson group proved this hypothesis to be incorrect (Cahan and Paulson, 1980; Fried et al., 1981). They showed that mPyV recognized specific sialic acid structures on red blood cells and on mouse cells containing α2,3-linked sialic acid. Small and large plaque binding variants of mouse polyomavirus were subsequently shown to have different affinities for a branched compound that additionally contained a second, α2,6-linked sialic acid. Large plaque strains bound less well to the branched structure than did the small plaque strain (Cahan et al., 1983). These data correlated well with an earlier study showing that small plaque viruses adsorb to cells much better than the large plaque strains (Diamond and Crawford, 1964).

In more recent studies from the Benjamin lab these differences were exploited and the plaque phenotypes genetically mapped to a single amino acid difference in the major capsid protein of the virus (Freund et al., 1991b). More importantly these differences in plaque behavior directly translated to their behavior in mouse models of infection (Dubensky et al., 1991; Freund et al., 1991a). The large plaque strain spread more efficiently in mice and induced a higher frequency of tumors and the tumors were more widespread than those caused by the small plaque strains. This led to the hypothesis that the branched structures act as pseudoreceptors that are bound by the virus, but do not result in productive infection. The analysis of virus–host cell interactions was thus important for understanding spread and pathogenicity of this group of viruses and generated increased interest in identifying specific receptors.

The Benjamin lab also attempted to isolate specific proteinaceous receptors for mPyV by producing and screening several thousand mononclonal antibodies raised against permissive mouse cell surfaces (Bauer et al., 1999). None of the antibodies were found capable of inhibiting infection leading them to conclude that no single molecule on the surface of the cells served as a specific receptor.

The first hint of a proteinaceous receptor for mPyV came from studies in the Amati lab where antibodies directed against natural integrin ligands as well as antibodies directed at α4β1 integrin inhibited a post-attachment step in infection (Caruso et al., 2003) (Table 1). This was found to be mediated by an LDV integrin binding motif present in mPyV VP1 as a specific mutation in this motif reduces

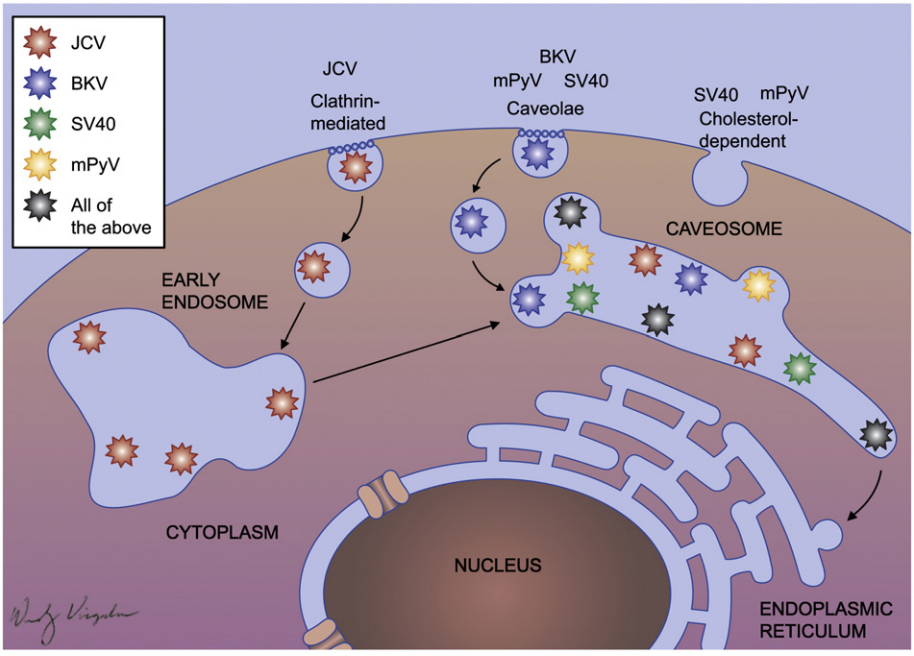

Fig. 2. General model of infectious entry pathways utilized by polyomaviruses. JCV is unique in that it initially enters cells by clathrin dependent endocytosis. The virus then traffics to early endosomes and then to caveosomes. BKV, SV40, and mPyV are reported to use caveolae dependent mechanisms of entry that traffic these viruses to caveosomes. SV40 and mPyV can also use non-caveolar but cholesterol dependent mechanisms of entry to access the caveosome and the mechanism used depends on cell type. Once in the caveosome SV40 and mPyV traffic to the endoplasmic reticulum in tubular structures that bud off the ER membrane. It is presumed that JCV and BKV use the same pathway but data on this is lacking. Adapted from Marsh and Helenius, 1989.

infectivity (Caruso et al., 2007). Interestingly, when inoculated into newborn mice, the mPyV displays an altered pattern of infection when compared to wild type (Caruso et al., 2007). Previous receptor hunting experiments described above would have missed this ligand as the virus apparently needs to undergo a conformational change after binding to sialic acid that then allows it to bind to the integrin receptors as a second step in the entry process. It is not certain whether infection of all cells requires this second step but it is clear that in some cases it is utilized. However, the LDV motif is buried under the receptor binding loops deep within VP1, and the mutation could have affected the structure of the binding site indirectly.

Recent studies from both the Benjamin and Rapoport groups clearly delineated a role for the gangliosides GD1a and GT1b in mPyV infection and for the ganglioside GM1 in SV40 infection (Gilbert and Benjamin, 2004; Gilbert et al., 2005; Tsai et al., 2003) (Table 1). The Rapoport group used sucrose flotation assays to initially ask whether mPyV would float when mixed with plasma membrane preparations from human erythrocytes, from bacterial membranes or from yeast membranes. They found that when mPyV was incubated with buffer or with either bacterial or yeast membranes the virus fractionated in the bottom third of the gradient. When mPyV was incubated with plasma membrane from human erythrocytes the virus floated to the top of the gradient indicating an association with plasma membrane lipids. Upon treatment of the plasma membranes with neuraminidase the virus failed to float and fractionated at the bottom of the gradient. Specific gangliosides were then tested for their ability to interact with polyomaviruses. mPyV only floated to the top of the gradient in the presence of GD1 and GT1b, and SV40 only floated to the top of the gradient in presence of GM1. The group then showed that rat cells cell deficient in complex ganglioside biosynthesis are poorly susceptible to mPyV and SV40 infection. However, when preincubated with GD1a or GT1b they become highly susceptible to mPyV and GM1 restores susceptibility to SV40. Fluorescent co-localization studies indicated that the virus traffics directly to the ER with the gangliosides (Fig. 2).

This mode of internalization is strikingly similar to the one used by ER directed bacterial toxins of the AB_5 family, such as cholera toxin, whose known receptors are gangliosides. In the case of the toxins, however, trafficking proceeds through the Golgi. Thus far there is no evidence that mPyV or SV40 traffics through the Golgi and instead they appear to traffic through caveosomes to eventually reach the ER where partial uncoating of the viruses take place. Once the mPyV reaches the ER it takes advantage of an unusual member of the protein disulfide isomerase family, ERp29 (Magnuson et al., 2005) (Fig. 2). PDI family members are oxidoreductases that contain one to several CXXC motifs in their thioredoxin domains (Hebert and Molinari, 2007). They function to both reduce and oxidize disulphide bonds in newly synthesized proteins. They also isomerize disulphide bonds assisting in the formation of native protein structures. ERp29 is structurally related to PDI proteins but it contains only a single cysteine in its

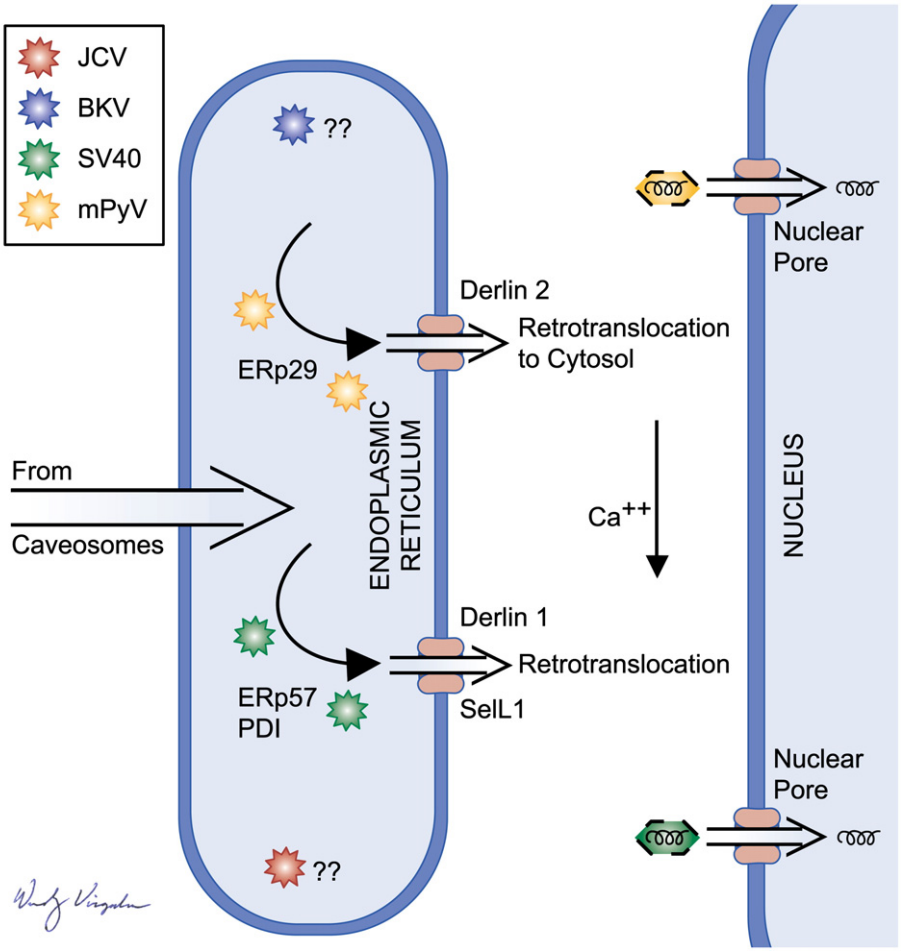

Fig. 3. Mechanisms used by polyomaviruses to exit the ER and target their genomes to the nucleus. The mouse polyomavirus and SV40 both traffic to the ER and it is presumed but not formally proven that JCV and BKV do the same. Once in the ER mPyV utilizes the unusual PDI family member, ERp29 to induce a conformational change in its coat protein. This may lead to cleavage of the C-terminal arm of VP1 allowing the particle to bind to lipid membranes. The ER retrotranslocation protein Derlin 2 is involved in ER exit. SV40 utilizes the canonical ER oxidoreductases, PDI, and ERp57, to unravel 12 of the vertex pentamers in the virus shell. This unfolded protein is then recognized by components of the ER associated degradation (ERAD) pathway (Derlin 1 and SelL1) to exit the ER. The viruses encounter a low calcium environment in the cytosol that may allow for their further disassembly. Once disassembled the viral protein–mini-chromosome complex is transported across an intact nuclear pore for genome delivery to the nucleus.

entire sequence and hence can only function in the isomerization reaction. A role for ERp29 in normal cellular functions has not been established. The Tsai group identified ERp29 as the PDI family member responsible for inducing a conformational change in mPyV VP1 that exposes a trypsin sensitive site in VP1 (Tsai et al., 2003) (Fig. 3). This conformational change is thought to result in the proteolytic cleavage of the C-terminal arm of VP1. As the C-terminal arm is involved in linking and stabilizing pentamer–pentamer interactions its cleavage would be predicted to destabilize the particle. The cleavage also led to the formation of a virion particle capable of binding and penetrating a lipid bilayer. This is predicted to facilitate the release of a partially disassembled and de-stabilized virion into the cytosol where nuclear localization signals in the VP1 protein could direct it across the nuclear pore (Fig. 3). Work from the Benjamin lab identified another ER protein as being critical for mPyV infection. In this study shRNAs directed at a protein involved in retrotranslocation of misfolded proteins out of the ER, Derlin 2, was found to significantly impair mPyV infection (Lilley et al., 2006) (Fig. 3). In related work Helenius and colleagues found that several ER associated factors played critical roles in SV40 disassembly and release from the ER (Schelhaas et al., 2007) (Fig. 3). Inhibition of PDI, ERp57, and Derlin 1 by siRNA knockdown all inhibited SV40 infection of cells. Knockdown of ERp29, calnexin or calreticulin had no effect on SV40 infection. They found that ERp57 uncoupled the 12 five-coordinated VP1 pentamers of SV40. This led to complete uncoating in the presence of EGTA, which removes the calcium ions that help to anchor the incoming C-terminal arms. The group also demonstrated a role for Derlin 1 and SelL1, two proteins involved in the ERAD retrotranslocation pathway in SV40 infection (Fig. 3). The model that emerges is one where the virion is delivered to the ER where it is recognized by components of the ER quality control machinery and is subjected to PDI related proteins that isomerize specific disulfide bonds involved in linking pentamers together within the capsid. The destabilized virion is then recognized essentially as a misfolded protein and is retrotranslocated out of the ER where it encounters a low calcium environment in the cytosol. Calcium is critical for viral particle stability and low levels of calcium in the cytosol likely contribute to further destabilization of the virion. Exposure of nuclearlocalization signals in viral capsid proteins that likely remain bound to the mini-chromosome then transport the mini-chromosome across the nuclear pore (Ishii et al., 1996; Kasamatsu and Nakanishi, 1998; Nakanishi et al., 1996; Yamada and Kasamatsu, 1993) (Fig. 3). It thus appears that at least two polyomaviruses, mPyV and SV40, utilize ER associated degradative pathways for uncoating.

SV40

Studies on SV40 entry mechanisms proceeded similarly and often in parallel with the work on mPyV described above. Hummeler and colleagues followed the fate of SV40 particles in cells by electron microscopy (Hummeler et al., 1970). They describe the majority of particles becoming rapidly enveloped at the plasma membrane into monopinocytotic vesicles. SV40 particles could be seen as early as 1 h post-infection in the nucleus. Maul and colleagues subsequently investigated the uptake and trafficking of SV40 in permissive and non-permissive cells (Maul et al., 1978). No qualitative differences were seen between the two cell types indicating that these steps did not restrict virus infection of cells. Their findings were very similar to those of Hummeler but they described several intervening steps in the entry process including a prominent association of viral particles in tubular membrane structures and of particles associated with rough ER. They did observe particles in the nucleus but these particles were not surrounded by membrane. They proposed a model where the entry of SV40 followed a series of membrane fusion and fission steps eventually liberating virions in the nucleus for uncoating. As they observed some free virions in the cytoplasm and some in the nucleus they proposed that the nucleus must contain an uncoating enzyme not present in the cytosol. As it turns out it appears that the ER contains the necessary factors for uncoating. The tubular structures they observed were likely virions pinching off in large tubular structures from caveosomes as described by Helenius many years later (Pelkmans et al., 2001). These tubular vesicles are transported to the ER in a microtubual dependent manner (Pelkmans et al., 2001) (Fig. 2). We now appreciate that most if not all polyomaviruses are trafficked to the ER where uncoating occurs or at least begins.

The single virus particles being enclosed in membranous vesicles at the plasma membrane described in the Maul paper were the first demonstration of caveolae which were only later appreciated as being critical organelles for SV40 entry into cells (Anderson et al., 1996) (Fig. 2). Caveolae are membrane microdomains rich in sphingolipids and signaling molecules. SV40 exploits these domains most likely by virtue of its interaction with the ganglioside GM1. Entry is relatively slow and proceeds in a pH neutral manner from caveolae to caveosomes to the ER (Pelkmans et al., 2001). In addition to the ganglioside GM1, SV40 was also reported to use MHC class I molecules to infect permissive cells (Atwood and Norkin, 1989) (Table 1). The virus can infect some cells lacking MHC I and in these cases GM1 is the principle receptor although infection is inefficient. The relationship between these receptors remains unclear.

JCV and BKV

The human polyomaviruses, JCV and BKV were discovered more recently and difficulties in isolating receptors for the mouse polyomavirus and SV40 probably precluded any interest in pursuing this with JCV and BKV as both are somewhat more difficult to propagate than SV40 or the mouse polyomavirus. With the introduction of new tools and techniques these issues have begun to be explored by several groups. Both of the human polyomaviruses require sialic acid to infect cells. JCV can use either an α2,3- or an α2,6-linked sialic acid to infect permissive glial cells (Dugan et al., 2007; Liu et al., 1998) and a ganglioside (GT1b) may also be involved (Komagome et al., 2002) (Table 1). BKV uses only the α2,3-linkage to infect cells (Dugan et al., 2005) (Table 1). A prominent role for the gangliosides GD1b and GT1b has been observed for BKV infection of kidney cells (Low et al., 2006) (Table 1).

BKV entry into cells is also similar to that of SV40, using caveolae mediated endocytosis to presumably deliver the virus to the ER for uncoating (Eash et al., 2004) (Fig. 2). JCV behaves differently than any of the described polyomaviruses and rather than being internalized directly into caveolae the virus utilizes clathrin dependent mechanisms to infect the cell (Pho et al., 2000) (Fig. 2). Virus entry proceeds by the normal clathrin dependent pathway to early endosomes (Querbes et al., 2004) (Fig. 2). Once in the early endosome, however, the virus is transported to caveosomes in a pH dependent manner (Querbes et al., 2006). From here the virus is most likely transported to the ER, presumably the common site of all polyomavirus uncoating events in the cell.

In addition to utilizing sialic acid as a receptor JCV has been shown to require the serotonin receptor, 5HT2aR, to infect glial cells (Fonseca-Elphick et al., 2004) (Table 1). The 5HT2a receptor is sufficient to confer susceptibility to cells lacking this receptor (HeLa and HEK293A). Infection of 5HT2aR expressing cells is still neuraminidase sensitive indicating a prominent role for cellular carbohydrates in infection. The 5HT2aR protein contains several potential glycosylation sites but it is unclear whether the sialic acid moieties responsible for infection reside on this protein or on other sialic acid containing molecules such as gangliosides. One group has found that JCV can infect human brain microvascular endothelial cells that lack the 5HT2a receptor (Chapagain et al., 2007). Infection is very inefficient but the results indicate that virus infection can proceed by alternative mechanisms on some cell types.

Structural studies on polyomaviruses and their interactions with carbohydrate

The polyomavirus capsid consists of 12 five-coordinated and 60 six-coordinated morphological units that assemble into a $T=7$d icosahedral capsid (Klug, 1965). Icosahedral capsid symmetry allows the virus to construct a container for its DNA from a large number of identical subunits, eliminating the need to encode many different components (Crick and Watson, 1956). Strict icosahedral symmetry, in which all subunits as well as their interactions with each other are identical, can only apply to capsids consisting of no more than 60 identical subunits. Some exceptionally simple isocahedral virus capsids are indeed constructed from 60 identical subunits and therefore possess strict icosahedral symmetry (Tsao et al., 1991). However, the need for more complex structures that can package larger genes as well as internal proteins requires for many viruses a capsid that cannot be formed with such a small number of subunits. Therefore, most viruses, including polyomaviruses, build larger capsids constructed from multiples of 60 subunits. In these capsids, subunits must be joined in a quasi-equivalent manner, meaning that they do not form the same contacts with their neighbors throughout the capsid, but exhibit flexibility or alternative binding modes to accommodate local symmetry mismatches (Caspar and Klug, 1962). The quasiequivalence principle postulated that the pentavalent and hexavalent morphological units of such larger icosahedral capsids were pentamers and hexamers of the same subunit. In some cases, this has been proven correct, e.g. for herpesvirus capsids (Zhou et al., 2000). The $T=7$d icosahedral capsids of polyomaviruses were therefore also thought to consist of 420 subunits, grouped into 12 pentamers and 60 hexamers of the major capsid protein VP1 (Finch, 1974). However, the crystal structure of the mPyV capsid at 22.5 Å resolution revealed that both the five- and six-coordinated morphological units of the capsid were in fact pentamers, a surprising result that appeared to challenge the concept of quasiequivalence as the same pentameric module is able to form both pentavalent and hexavalent contacts (Rayment et al., 1982). The crystal structure of SV40 at 3.8 Å resolution, which was determined almost 10 years later finally provided the molecular basis for the unexpected capsid construction of polyomaviruses (Liddington et al., 1991). It revealed that the interpentamer contacts are primarily mediated by C-terminal extensions of VP1, termed arms, that protrude in different directions from each VP1 monomer and contact neighboring pentamers. Both pentavalent and hexavalent pentamers donate and accept five C-terminal arms to tie the capsid together. The VP1 pentamer cores and the contacts between incoming arm and acceptor monomer are identical throughout the capsid, demonstrating that the polyomavirus capsid also uses a form of quasiequivalence as the basis for its construction. Monomeric VP1 is formed by two antiparallel β-sheets: a sheet formed by strands B, I, D and G (the BIDG sheet) and a sheet formed by strands C, H, E and F (the CHEF sheet). The two β-sheets are organized into a jelly-roll fold, a structural motif that is quite often observed in virus capsid proteins but rare in non-viral proteins. Each incoming arm adds one strand (J′) to the edge of the BIDG sheet of a monomer. The J′ strand is then fixed by the N-terminus of the accepting monomer, which acts as a clamp by adding a short helix and a final β-strand (A) to the sheet next to the J′ strand. The assembled structure is further stabilized by two calcium ions. These findings were confirmed for mPyV and are thought to hold true for the other members of the polyomavirus family as well because of conservation of the key contact residues (Stehle et al., 1996; Stehle et al., 1994).

Structural studies of unassembled, or "free", pentamers that lack their C-terminal arms provide insight into one aspect of capsid assembly (Neu et al., 2008; Stehle and Harrison, 1997). The only structural change between free VP1 and VP1 in the context of the capsid is a rearrangement at the N-terminus, which forms a helix fixing the incoming arm in the capsid. In free pentamers, this part of VP1 forms a β-strand that aligns with the G strand and contacts residues at the beginning of the C-terminal arm. One likely interpretation of this finding is that this arrangement serves to guide the C-terminal arm away from its own subunit, thereby preventing misassembly of the polyomavirus capsids.

Receptor binding by mPyV was structurally investigated early on for whole virions as well as for VP1 pentamers (Stehle et al., 1996; Stehle and Harrison, 1997; Stehle et al., 1994) as it was known that mPyV bound α2,3-linked sialic acid (Cahan et al., 1983; Fried et al., 1981). The sialylated oligosaccharides used as receptor mimics bound to VP1 in shallow pockets on the outer surface of the pentamers. These sialylated oligosaccharides were later found to be portions of the GD1a and GT1b gangliosides, which are the physiologic receptors for mPyV (Tsai et al., 2003). The same group also identified the ganglioside GM1 as a receptor for SV40 (Tsai et al., 2003). The structural and functional characterization of the interaction between SV40 and GM1 was made possible because a wide variety of glycans were available for defining receptor specificity and as the oligosaccharide portion of GM1 was available in amounts sufficient for structural analysis (Campanero-Rhodes et al., 2007; Neu et al., 2008). As an increasing amount of structural information on viral attachment proteins in complex with sialylated carbohydrate receptors has become available in recent years, we will focus the remainder of this chapter on those findings and their general implications.

Viral attachment to sialic acid

Sialic acids are present on virtually every cell type in higher vertebrates (Varki, 2008). Sialylation is a terminal modification on glycolipids and glycoproteins, and sialic acids can be attached to a variety of monosaccharides via different glycosidic linkages. Physiological functions include cell–cell attachment via specific receptors, general charge repulsion of blood cells as well as roles in neuronal plasticity, immune regulation and glomerular filtration (reviewed in (Varki, 2008). Many pathogens attach to sialic acid-containing receptors to enter cells, such as a number of viruses as well as bacterial toxins belonging to the *Clostridium* neurotoxin and AB_5 families.

The most common sialic acid in humans is N-acetyl neuraminic acid (NeuNAc). It forms a six-membered glycosidic ring, to which several groups are attached, some in substitution for the typical hydroxyl groups of carbohydrates: a carboxylate group at the anomeric carbon C2, an N-acetyl group at the C5 carbon, and a glycerol chain at carbon C6 (Fig. 4A). The overall structure of NeuNAc is rather rigid. In solution, the sugar ring prefers the energetically most favorable chair conformation, positioning its three distinctive groups in three different directions that serve as "handles" with which attachment proteins can interact. While the glycerol chain with three freely rotating bonds is quite flexible, the other two groups possess limited degrees of rotational freedom.

High-resolution structural information on the interaction of viral attachment proteins with sialylated carbohydrates is available for the following systems: Influenza virus A haemagglutinin (HA) in complex with oligosaccharides containing α2,3-linked and α2,6-linked NeuNAc (Eisen et al., 1997; Gamblin et al., 2004; Ha et al., 2001; Ha et al., 2003; Russell et al., 2006; Sauter et al., 1992; Stevens et al., 2006; Stevens et al., 2004; Weis et al., 1988), mPyV VP1 with a fragment of ganglioside GD1a (Stehle and Harrison, 1997), rhesus, swine and human rotavirus VP8* with methyl-α2,3-sialoside (Blanchard et al., 2007; Dormitzer et al., 2002a), adenovirus Ad37 fiber knob (Ad37) with α2,3- and α2,6-sialyllactose (Burmeister et al., 2004) and SV40 VP1 with ganglioside GM1 (Neu et al., 2008). Paramyxoviruses bind to their host cells with their haemagglutinin–neuraminidase protein that uses the same binding site for attachment to NeuNAc and cleavage of its glycosidic bond (Crennell et al., 2000). We will, however, focus here on viral proteins that solely

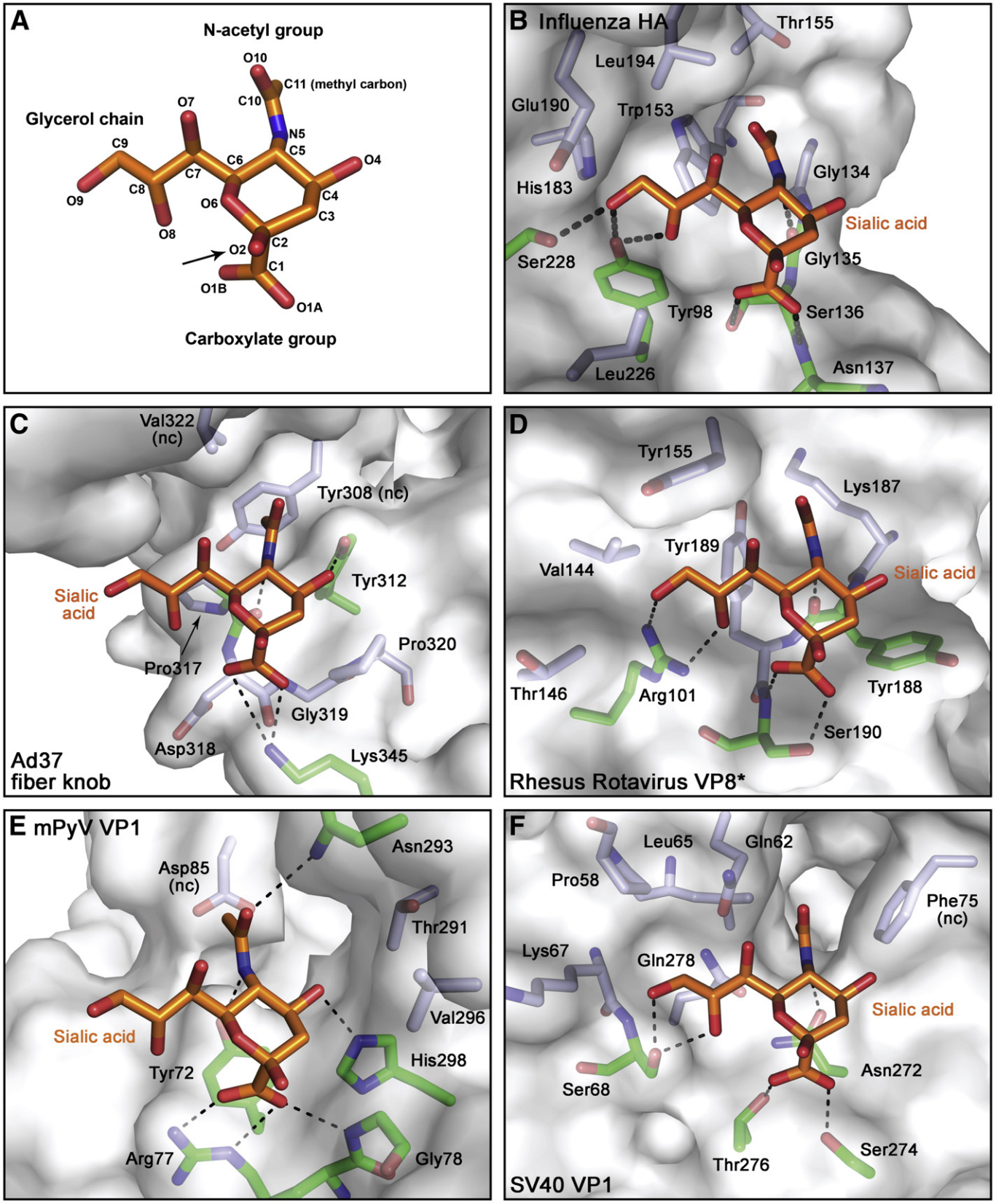

Fig. 4. Sialic acid recognition by viral attachment proteins. (A) Schematic of N-acetyl neuraminic acid. The arrow points to the O2 oxygen that is involved in glycosidic linkages to adjacent sugars. (B–F) Contacts of NeuNAc with Influenza HA (B), Ad37 (C), Rotavirus VP8* (D), mPyV VP1 (E) and SV40 VP1 (F). pdb entries 1hgg (B), 1uxa (C), 1kqr (A, D), 1vps (E) and 1bwr (F) were used to create this figure. NeuNAc is depicted in orange, the protein residues forming hydrogen bonds to NeuNAc are colored green and the residues making van der Waals contacts are colored grey. The viral surface is shown in grey. This figure was prepared with PyMol (DeLano Scientific, Inc.).

mediate attachment and do not possess enzymatic activity. Sialic acid binding by catalytically active viral neuraminidases is probably not directly comparable to binding by attachment proteins, as neuraminidase function requires high affinity as well as additional binding energy to distort the sialic acid for catalysis. Neuraminidases thus feature deeply buried binding sites with a higher number of specific contacts than are found in attachment proteins.

Although most of the attachment proteins listed above are not homologous and are found on unrelated enveloped as well as non-enveloped viruses, there are some general similarities in the modes of oligosaccharide binding. First, in all viral complexes, NeuNAc is the major, and in the case of Ad37 and VP8* the only, point of contact. This distinguishes these complexes from, for example, bacterial toxins, which can also recognize sialylated receptors but form more contacts with carbohydrates attached to sialic acid than with the sialic acid itself (Fotinou et al., 2001; Merritt et al., 1994; Stenmark et al., 2008). Second, all viral attachment complexes bind NeuNAc in shallow depressions rather than deep grooves on the protein surface. The affinities for these interactions are only in the millimolar range, which is consistent with the exposed binding surfaces (Burmeister et al., 2004; Dormitzer et al., 2002b; Eisen et al., 1997; Neu et al., 2008; Stehle et al., 1996). A relatively low affinity may in fact be crucial for a productive viral infection as a very tight attachment to the host cell will prevent progeny virus from spreading. Third, the attachment protein complexes all feature NeuNAc in its chair conformation, which is the preferred conformation in solution. Fourth, as detailed in the next section, there are several parallels in the strategies used by attachment proteins to bind sialic acid.

The listed similarities observed in complexes of viral attachment proteins and sialylated receptors do not extend to other proteins that also bind the same type of receptors, indicating that they do represent characteristic properties of viral proteins that mediate engagement of host cell receptors. For example, the low affinity binding by viral attachment proteins is in contrast to bacterial toxins, which also bind sialylated oligosaccharides but employ deeper pockets and generally feature significantly higher affinity (Fotinou et al., 2001; Merritt et al., 1994; Stenmark et al., 2008). In contrast to the numerous binding sites on viruses, bacterial toxins have only one or at most five carbohydrate binding sites, and the interactions between these and cellular receptors must be sufficient to provide cell entry.

Specific contacts with sialic acid

The charged carboxylate group of sialic acid is arguably the most uniquely identifiable feature of this carbohydrate when compared to similar, uncharged structures. It is therefore perhaps not surprising that all of the viruses or viral proteins investigated here engage this group. However, they do so by employing two different strategies. The mPyV and Ad37 form salt bridges from the positively charged side chains of Arg and Lys residues to the negatively charged carboxylate group (Figs. 4E and C, respectively). By contrast, the other three viruses engage the carboxylate group with two parallel hydrogen bonds. In each case, the carboxylate group accepts two hydrogen bonds from two specifically spaced hydrogen bond donors in the proteins. In the Influenza HA, SV40 VP1 and Rotavirus VP8* complexes, these are either the hydroxyl groups of Ser and Thr side chains or backbone amido groups (Figs. 4B, D, E). These parallel hydrogen bonds also have similar geometry. For each of them, the angle between the carboxylate C1, the carboxylate O that is engaged and the hydrogen bond donor approaches 120°.

Recognition of the negatively charged carboxylate group of NeuNAc alone would not distinguish it from other molecules bearing a negative charge. The N-acetyl group, situated on the opposite side of the sugar ring as the carboxylate group, can serve as additional “handle” on NeuNAc and it provides specificity by additional favourable contacts with the correct ligand. The N5 atom of the N-acetyl group is contacted by a hydrogen bond in each of the complexes. The binding partners are hydrogen bond acceptors in the protein, such as backbone carbonyl groups (Figs. 4B–D), the side chain carbonyl group of an Asn residue (Fig. 4F) or the hydroxyl group of a Tyr side chain (Fig. 4E). The mPyV VP1 also forms an additional hydrogen bond by an Asn side chain, which contacts the carbonyl group of the acetyl function. With the sole exception of mPyV VP1, the binding sites of all proteins also feature a depression into which the methyl group of the N-acetyl moiety inserts. This depression is shallow and rather hydrophobic in the case of Ad37, Influenza HA and Rotavirus VP8*, as it is lined with hydrophobic side chains (Figs. 4B–D). Interestingly, SV40 VP1 does not feature such a shallow depression to accommodate the methyl group. Rather, it has a substantially deeper pocket that is formed by hydrophobic residues such as Leu65 and Phe75 and lined with hydrophilic side chains of residues Gln62 and Gln278 at its rim (Fig. 4F). The natural hosts of SV40 are monkeys, in which N-glycolyl neuraminic acid (NeuNGc) is the predominant sialic acid. In contrast to the human NeuNAc, NeuNGc has an additional hydroxyl group attached to the methyl group. The big, partly hydrophilic and likely water-filled cavity in SV40 could likely accommodate the more polar NeuNGc better than NeuNAc (Campanero-Rhodes et al., 2007), which might account for its shape.

Even though contacts with the carboxylate and N-acetyl groups are sufficient to position NeuNAc correctly on the protein surface, most attachment complexes feature contacts with the polar and flexible glycerol moiety. This group protrudes away from the protein in the mPyV VP1 and Ad37 complexes (Figs. 4C and E), but is recognized by a number of contacts in the other complexes. There are hydrogen bonds to O8 and O9 by each of SV40 VP1, Influenza HA and Rotavirus VP8* that are formed by Ser, Tyr and Arg side chains (Figs. 4B, D, F). In addition, the glycerol chain lies in a shallow pocket in all of these complexes and makes van der Waals contacts to residues that confer shape specificity.

In summary, while all of the binding sites recognize the carboxylate group and form a hydrogen bond to the N-acetyl group, only some (SV40 VP1, Influenza HA and Rotavirus VP8*) contact the glycerol chain both by hydrogen bonds and shape complementarity. In addition, most also feature a depression that accommodates the methyl group of the N-acetyl moiety. Since all binding surfaces are exposed, the interactions determining NeuNAc binding lie in the rather polar environment of the protein surface. The polar interactions are therefore probably quite weak. Hydrogen bonds between sialic acid and protein atoms can be replaced by contacts between water molecules and protein atoms without much difference in energy. Salt bridges in aqueous solution also possess less energy than salt bridges that are located in a partially hydrophobic, solvent-inaccessible environment. Likewise, the non-polar van der Waals interactions between carbohydrate and protein are also not very strong as such interactions increase in strength with the area of surface buried in the contact. Most of the contacts described above exclude rather small areas from solvent compared to the extensive interfaces found in protein–protein complexes.

The observed, relatively weak interactions are consistent with the experimentally-determined millimolar affinities for the protein–glycan complexes. The shallow binding sites of attachment proteins do not exclude binding of incorrect ligands by steric clashes. Instead, specificity is provided by favorable interactions that are spaced in a way that only the correct ligand can engage in all contacts. In the case of Influenza HA, mPyV VP1 and SV40 VP1, sialic acid by itself is not sufficient for binding, but additional sugars are also needed for the interaction.

How do viruses discriminate among sialic acids in different contexts?

Two of the viruses mentioned before, Rhesus Rotavirus and Ad37, appear to bind NeuNAc relatively indiscriminately, although it is

possible that additional carbohydrates may contribute to binding. mPyV, SV40 and different strains of influenzaviruses, however, have distinct specificities for the structural contexts of the sialic acid.

mPyV VP1 binds carbohydrates containing a NeuNAc-α2,3-Gal motif that is unbranched at the Gal position (Cahan et al., 1983). The Gal moiety is recognized by a hydrogen bond between the backbone carbonyl group of Gly78 and the distinctive axial O4 hydroxyl group of Gal (Stehle and Harrison, 1997). The lack of a side chain in Gly78 also confers binding specificity since the presence of even a small side chain in this position would cause sterical clashes with the Gal and abolish binding. The architecture of the binding site accepts only one conformation of the NeuNAc-α2,3-Gal glycosidic linkage. This conformation is stabilized by an internal hydrogen bond and also occurs in solution, but it could not be adopted by compounds that are branched at the Gal residue due to steric clashes.

SV40 VP1 possesses quite narrow specificity for the oligosaccharide part of the ganglioside GM1, which is a branched compound of the following structure: Gal-β1,3-GalNAc-β1,4-[NeuNAc-α2,3-]-Gal-β1,4-Glc. While SV40 still binds the same compound with NeuNGc substituted for NeuNAc, binding is abolished if one of the terminal sugars, i.e. Gal or NeuNAc, are removed or if one of them bears an additional sugar moiety (Campanero-Rhodes et al., 2007; Neu et al., 2008). Both the Gal-β1,3-GalNAc and NeuNAc branches directly contact the protein, providing binding affinity. The core structure of GM1, i.e. the branching Gal with GalNAc and NeuNAc attached to neighboring atoms is sterically constrained as clashes between GalNAc and NeuNAc could occur in many conformations of the glycosidic linkages. The moiety adopts one dominant conformation in solution (Poppe et al., 1994), which is bound by SV40 VP1. Specificity therefore arises from SV40 recognizing the correct placement of the two binding arms by a rigid spacer.

Different strains of Influenza A virus infect different species because their HA molecules bind sialic acid in different contexts. The adaptation of avian HAs to human hosts occurs by a switch in binding specificity from avian-like α2,3-sialylated glycans to the longer α2,6-sialylated glycans that are present in the upper respiratory tract epithelia of humans (Russell et al., 2006; Skehel and Wiley, 2000; Stevens et al., 2006; van Riel et al., 2007). NeuNAc is bound in the same orientation and by the same residues of both avian and human HAs so that specificity is conferred by the residues in contact with additional monosaccharides. Recent data indicate that the different glycan types are not discriminated by contacts with the sugar residue to which the NeuNAc is linked, but that the different overall topology of the glycans is recognized (Chandrasekaran et al., 2008). Given the preferred torsion angles of the NeuNAc-α2,3-Gal-β1,3/4-GlcNAc motif typical of the recognized α2,3-linked glycans, the Gal-β1,3/4-GalNAc part is fairly linear and can occupy the space delineated by a cone with NeuNAc at its tip. Sugar moieties farther away from the NeuNAc mostly do not interact with the protein in this conformation. The glycans containing α2,6-linked NeuNAc, for which NeuNAc-α2,6-Gal-β1,4-GlcNAc is typical, can also adopt this cone-shaped topology. However, these also sample a topology, in which the Gal is relatively fixed with respect to NeuNAc and there is a kink at the Gal position, forcing the GlcNAc and subsequent sugars to point back towards the protein. The space that can be sampled by the glycan therefore resembles an umbrella, with NeuNAc-α2,6-Gal as the stick and the rest of the glycan as the umbrella. For α2,6-linked trisaccharides that can sample both cone- and umbrella-shaped topologies, the cone-shaped one is preferred. However, the longer α2,6-linked glycans that confer infectivity in humans prefer the umbrella-shaped topologies, leading to additional contacts with the protein. If the HA of an influenza strain cannot accommodate these long, umbrella-shaped α2,6-linked glycans, it cannot spread in humans (Chandrasekaran et al., 2008).

Several of the structural and functional studies described above required the use of correct ligands. The use of model glycans such as α2,6-linked sialyllactose would not have revealed the difference in topology that is crucial for glycan recognition by HAs, as these short glycans do not adopt the umbrella shape that is bound by human influenzaviruses. In the case of SV40, only the correct GM1 receptor bound to the protein. Smaller, unbranched components of the GM1 structure would not have given insight into SV40 receptor binding as they do not bind with detectable affinity (Neu et al., 2008). Several other viruses, e.g. strains of coronavirus and reovirus, bind sialylated glycans, but structural information about these interactions is currently lacking. The availability of complex glycans and the use of glycan screens to define binding specificity have proven of great importance and will undoubtedly advance the understanding of glycan binding by these other viruses.

Discussion

The original studies describing polyomavirus host cell interactions depicted infection as involving a series of discrete steps that included virus adsorption, virus entry into membrane bound organelles, and virus trafficking by sequential membrane fusion and fission events. Although there was controversy regarding whether these viruses could penetrate the nuclear envelope intact an early pivotal study demonstrated that the virus capsid only penetrates the outer nuclear envelope. Recent studies on these viruses describe largely the same series of steps but in significantly more detail. We now know and appreciate that the majority of these viruses recognize specific linkages of sialic acid on gangliosides and glycoproteins. These viruses also recognize other cellular components such as the 5HT2a receptor for JCV, α4β1 integrin for mPyV, and the MHC class I protein for SV40, likely as part of a receptor complex with gangliosides or other sialic acid containing structures. There are clear cell type specific and viral strain differences that determine what components of the receptor complex are required to initiate infection. Once bound to cells all of these viruses induce molecular signals that initiate infectious entry. The mPyV, SV40, and BKV all infect cells by caveolae dependent endocytosis. Non-caveolar cholesterol dependent mechanisms can also be exploited by some of these viruses depending on cell type. Downstream of both caveolae and raft dependent mechanisms the viruses traffic to a pH neutral caveosome that lacks markers of other cellular organelles. High resolution video microscopy of virus entry has found that these viruses are trafficked out of the caveosomes in elongated tubular structures that fuse with the ER membrane. This trafficking step is dependent on microtubules. The human polyomavirus, JCV, appears to be an exception and rather than utilizing caveloae to enter cells this virus exploits clathrin dependent mechanisms to initially traffic to early endosomes. In the early endosomes JCV is found to be tightly opposed to the inner leaflet of the endosome and in a pH and Rab5 dependent step traffics from the early endosome to caveosomes. At this point the virus likely follows the fate of other polyomaviruses and travels to the ER. Once in the ER the viruses take advantage of protein disulfide isomerases to rearrange their capsid structure. In the case of SV40, PDI and ERp57 rearrange the 12 five-coordinated VP1 pentamers. The now misfolded structure is recognized by components of the ERAD pathway (Derlin 1, SelL1) and exported to the cytosol. Calcium levels in the cytosol of resting cells are significantly lower (0.1 μM) than levels in the ER (100–400 μM). As calcium is required to stabilize polyomavirus virions the low levels of calcium encountered as the destabilized virus exits the ERAD pathway are likely sufficient to further distort and disassemble the virion and prepare it for transport through the nuclear pore. In the case of mPyV a novel PDI like protein, ERp29 acts to unfold VP1 exposing a protease sensitive site in the C-terminal arm of the protein. Once cleaved the destabilized particle can insert into a lipid membrane. The virus may directly escape the ER via this mechanism or it might utilize the retrotranslocator Derlin 2 as inhibition of this protein reduces mPyV infection. It is likely that the

two human polyomaviruses use similar mechanisms to escape the ER but this has not been formally demonstrated.

Early structural studies on mPyV and SV40 challenged the quasi-equivalence theory of capsid assembly as there were 72 VP1 pentamers arranged that formed both pentavalent and hexavalent contacts. Subsequent high resolution structural studies provided the molecular basis for this unexpected capsid construction. It revealed that the interpentamer contacts are primarily mediated by C-terminal extensions of VP1, termed arms, that protrude in different directions from each VP1 monomer and contact neighboring pentamers. Both pentavalent and hexavalent pentamers donate and accept five C-terminal arms to tie the capsid together. The VP1 pentamer cores and the contacts between incoming arm and acceptor monomer are identical throughout the capsid, demonstrating that the polyomavirus capsid also uses a form of quasiequivalence as the basis for its construction. Recent high resolution structural studies on viral capsid proteins in complex with receptor fragments has provided a detailed picture of how these viruses engage this component of the receptor complex. This level of detail may provide the basis for the design of small molecular weight compounds capable of antagonizing polyomavirus host cells receptor interactions and may in time lead to novel therapeutic approaches to treat patients with polyomavirus induced disease.

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Further reading

Please see the manuscript entitled "Structure, attachment and entry of polyoma- and papillomaviruses" in this issue, which also describes polyoma virus structure and entry.

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