Review

Adenoviruses: update on structure and function

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Adenoviruses have been studied intensively for over 50 years as models of virus–cell interactions and latterly as gene vectors. With the advent of more sophisticated structural analysis techniques the disposition of most of the 13 structural proteins have been defined to a reasonable level. This review seeks to describe the functional properties of these proteins and shows that they all have a part to play in deciding the outcome of an infection and act at every level of the virus's path through the host cell. They are primarily involved in the induction of the different arms of the immune system and a better understanding of their overall properties should lead to more effective ways of combating virus infections.

Prologue

Adenoviruses (Ads) are relatively promiscuous in their ability to infect a wide range of species and tissues, but their propensity to produce disease is normally well circumscribed and is a function of the effective defences that are mounted by the infected host. In this review I shall concentrate on the structural characteristics of human adenoviruses and how they influence the outcome of disease.

There are 51 human Ad serotypes classified originally on the basis of their ability to be neutralized by specific animal antisera. These can be further subdivided into six species – or subgroups – (A to F) based on their capacity to agglutinate erythrocytes of human, rat and monkey as well as on their oncogenicity in rodents. Sequence availability has also allowed more detailed phylogenetic analysis to be employed in classification (Crawford-Miksza & Schnurr, 1996; Fauquet *et al.*, 2005). Species B is further subdivided into B1 and B2 (Segerman *et al.*, 2003a). There is some correlation between the species and their tissue tropism and clinical properties. Thus species B1, C and E mainly cause respiratory disease, whereas species B, D and E can induce ocular disease. Species F is responsible for gastroenteritis and B2 viruses infect the kidneys and urinary tract (see Table 22, in Russell, 2005).

Animal model systems (mouse, rat, dog, hamster and pig) have been explored for adenovirus respiratory disease (Ginsberg, 1999; Jogler *et al.*, 2006; Ternovoi *et al.*, 2005; Weinberg *et al.*, 2005; Ginsberg *et al.*, 1999; Toth *et al.*, 2005; Thomas *et al.*, 2006). A human lung organ culture has also been claimed to be a useful pointer to the *in vivo* infection (Booth *et al.*, 2004) and a recent report has demonstrated that a mouse model for keratitis can be constructed using human adenovirus 37 (Chintakuntlawar *et al.*, 2007). A number of studies have also examined experimental infection of healthy humans with a range of adenoviruses (Lichtenstein & Wold, 2004) and it was concluded that infection did not, in general, cause severe disease. Thus, when inoculated intranasally most Ads caused mild respiratory disease, but inhaled Ad4 produced acute respiratory disease (ARD). Very recent reports from the USA and Canada have implicated Ad14 (of species B) in episodes of ARD. A number of adenovirus species (Ads 1, 3, 4, 5, 8, 16, 26 and 27) produced conjunctivitis when swabbed into the eye.

These observations were consistent with the idea that the host defence mechanisms, for the most part, were functioning effectively in the healthy individual. Nevertheless, it is also evident that some serotypes can bring about considerable morbidity (e.g. ARD), especially in individuals who are compromised immunologically (e.g. transplant patients) or nutritionally (e.g. gastrointestinal infections in children in the developing world). In addition, with the very considerable interest in the development of adenoviruses as gene vectors, it is becoming ever more important to understand the molecular aspects of infection and the host response to it. In this review I shall update (Russell, 2000) the role of the structural components of the virus in defining the outcome of an infection.

The structure of the virus

With the progress made in X-ray crystallography and cryo-electron microscopy image reconstruction now providing resolution to 6 Å (0.6 nm), a better understanding of the disposition of a number of the structural components is evident (Fabry *et al.*, 2005; Saban *et al.*, 2005, 2006; Stewart *et al.*, 1993). Fig. 1 provides a schematic diagram of the current state of our knowledge of the complex adenovirus icosahedral capsid. The principal component is the homotrimeric hexon and there are 240 on the faces and edges of the capsid with the pentons consisting of the

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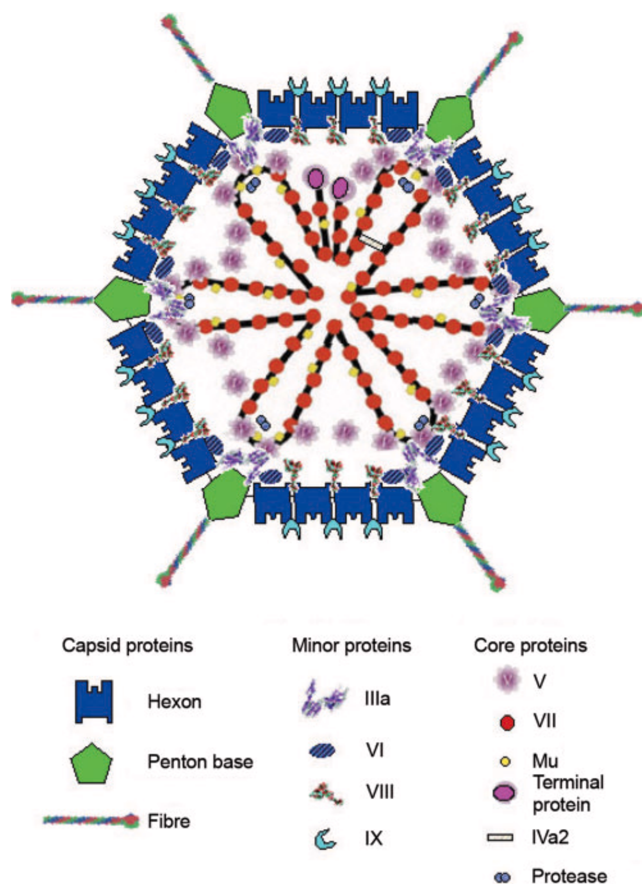

Fig. 1. Structure of adenovirus. A schematic depiction of the structure based on cryo-electron microscopy and crystallography. The locations of the capsid and minor components are reasonably well defined and are not to scale. The disposition of the core proteins and the virus DNA is largely conjectural. The symbols for IIIa and VIII are based on the structures defined by Saban *et al.* (2006).

penton bases and extended fibres on the 12 fivefold apices. Other so-called 'minor' components: IIIa, VI, VIII and IX are also associated with the capsid (Vellinga *et al.*, 2005). There are six other structural components situated in the virus core, five are associated with the double stranded DNA genome [V, VII, Mu, IVa2 and the terminal protein (TP)], the remaining component is the 23K virion protease which plays a vital role in the assembly of the virion (see below). Most of the detailed structural analyses have been carried out using human serotypes, although a recent study of canine adenovirus 2 has indicated that, while the basic features are retained, the capsid of the canine virus is much smoother and the fibre is more complex (Schoehn *et al.*, 2008). A recent structural analysis of an atadenovirus by cryo-electron microscopy has indicated that there are some differences from mastadenoviruses in capsid topology, but the main characteristic adenovirus morphology is retained (Pantelic *et al.*, 2008). A more detailed description of these structural proteins is given below as a forerunner for consideration of their role in infection.

Hexon

The hexon capsomere is a pseudo-hexagonal trimer situated on the 20 facets of the icosahedral capsid created by threefold repetition of two β-barrels at the base of each hexon molecule. The pseudo-hexagonal base allows close alignment within the facets and there are three tower regions that are presented to the exterior. There are 240 hexons in the capsid. Because of their different environments there are four kinds of hexon – designated H1, H2, H3 and H4 (Burnett, 1985). Sixty H1 hexons associate with the pentons at the 12 apices and are also termed peripentonal hexons (Fig. 2a). The remaining hexons are designated 'groups of nine' or GONs on the 20 faces of the icosahedron and are further defined as H2 (on the twofold axes), H3 (on the threefold axes) and the remaining ones as H4 (Fig. 2a).

The size of the hexon molecule can vary with the serotype – the largest described is from Ad2 and comprises 967 aa. Up to nine hypervariable regions, determined by comparative sequence analysis of serotypes, are present in each hexon molecule. These are situated at the top of the molecule and six can be resolved as α-helical rods in the 6 Å structure (Saban *et al.*, 2006). These relate to the type-specific antigens of the hexon and at least one of them constitutes the major part of the virus-neutralizing activity (Crawford-Miksza & Schnurr, 1996; Pichla-Gollon *et al.*, 2006; Roberts *et al.*, 2006; Rux *et al.*, 2003; Takeuchi *et al.*, 1999) (Fig. 3a).

The base of each hexon molecule has one loop and two eight-stranded 'jelly rolls' which provide the means for interacting with neighbouring capsomeres, probably via charged residues in interacting loops. There must be considerable flexibility in these interactions given the differing environments of the H1 to H4 hexons. The N and C termini lie beneath the base and do not seem to take part in interactions with other hexons.

Penton

The penton capsomere is a covalent complex of two proteins – the homopentameric penton base and the homotrimeric fibre protein protruding from the 12 vertices of the icosahedron (Fig. 1). The fibre has three distinct regions: tail, shaft and knob.

The penton base monomer in Ad2 comprises 471 aa and its pentameric structure (Fig. 3b) has been determined to 3.3 Å (0.33 nm; Zubieta *et al.*, 2005) and consists of two domains: the lower one with the typical jelly roll of two four-stranded anti-parallel β-sheets forming a β-barrel, and the upper one with irregular folds formed by two insertions arising from the lower jelly roll strands. The first insertion contains the RGD loop (discussed later) and the other a loop which is variable between serotypes. Pentamerization can occur, providing stability by the burying of hydrophobic surfaces. A pore occurs along the fivefold axis of the pentamer and the top narrow part is predominantly hydrophobic. The β-barrels from the surrounding peri-

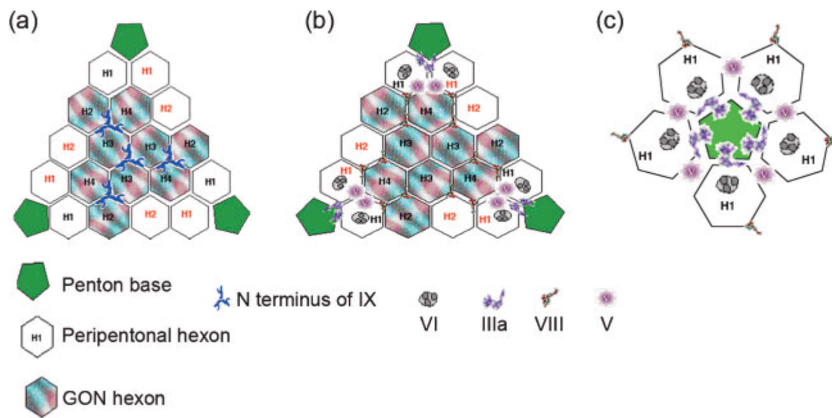

Fig. 2. Facets of the adenovirus icosahedron. (a) External. The GON hexons are multicoloured and the H1 peripentonal hexons are either lettered in black when they are on the same plane as the GONs or lettered in orange where they are associated with GONs on a different facet. Similarly, the H2 hexons lettered in orange are associated with GONs on a different facet. The symbol for protein IX is not to scale. (b) Internal. Hexons are designated in (a). Note symbols for other structural proteins are not to scale. (c) Internal structure at the apex; symbols as above.

pentonal hexons appear to interact with grooves in the penton base.

The fibre polypeptide comprises 582 aa in Ad2 and binds non-covalently by its N terminus onto the top surface of the penton base (Devaux *et al.*, 1987; Zubieta *et al.*, 2005). It is found that a sequence near the N terminus (FNPVYPY), which is highly conserved between serotypes (Tarassishin *et al.*, 2000), lies in a relatively hydrophobic groove on the top surface of the base formed between two adjacent monomers (Zubieta *et al.*, 2005). There are also a number of hydrogen bonds and a salt bridge contributing to the stability of the interaction with the penton base. The peptide sequences taking part in this interaction are also well conserved between serotypes. The symmetry mismatch here appears to be a function of co-operative conformational changes following fibre binding, with the result that there is room for only the three conserved fibre peptides. These are arranged horizontally and radially on the base, allowing the three flexible tails to form the characteristic protruding trimeric fibre. The fibre polypeptide consists of a variable number of pseudorepeats of 15–20 aa connected by a β-turn (Green *et al.*, 1983). These repeats form a shaft of three intertwined strands (van Raaij *et al.*, 1999) that is rigid and stable, but of varying length depending on the number of pseudorepeats (related to the serotype). However, there can be disruptions to the shaft sequences, allowing the fibre to form hinge regions (Chroboczek *et al.*, 1995). A further ~180 aa form the C-terminal globular head or knob. Trimerization is governed by sequences in both the knob and shaft regions (Li *et al.*, 2006). The knob contains eight-stranded β-barrels in each subunit and has a central depression with three symmetry-related valleys. There are a number of loops emanating from the knob (designated DG, HI and AB) (Xia *et al.*, 1994) and these will be discussed below in the light of their role in receptor

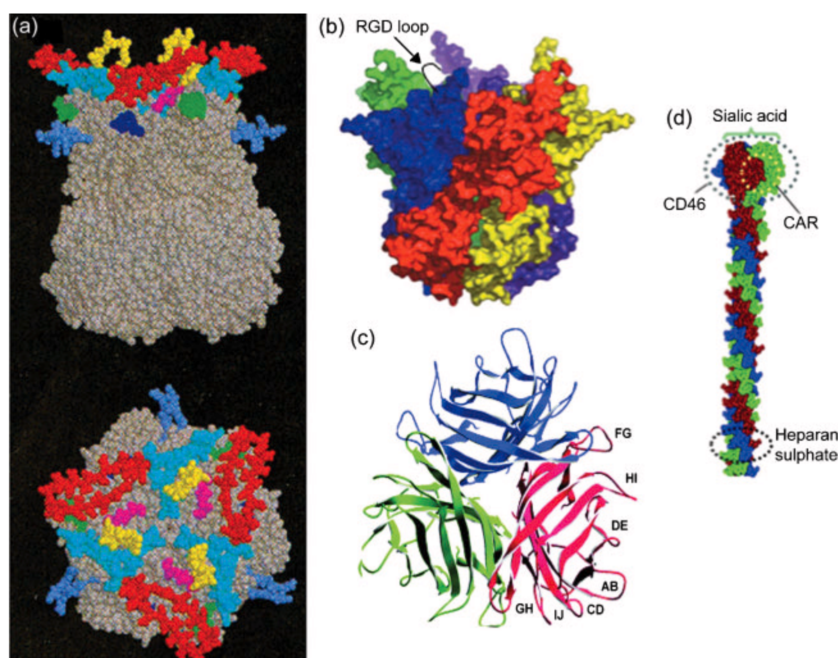

Fig. 3. Structure of the capsomeres. (a) Ad5 hexon trimer space-filling model showing seven hypervariable loops in colours with the remainder of the hexon in grey. Top shows side view and bottom the view from the top of the hexon. (b) Penton base from cryo-electron microscopic image reconstruction of the Ad5 capsomere: side view showing one of the RGD loops from the pentameric structure. (c) A ribbon representation of the fibre knob showing protruding loops with the agreed notation. All fibre knobs have the same general structure with variations in the number and amino acid composition. This one is from Ad35. (d) Fibre space-filling model indicating sites for receptor attachment modelled from the atomic structure of Ad2. The figures above were taken with permission from Roberts *et al.* (2006) for (a), Zhang & Bergelson (2005) for (b) and (d) and from Wang *et al.* (2006) for (c).

recognition (Fig. 3c). Glycosylation of the fibre shaft at a serine residue has been reported, although it is not clear if this has a functional role (Cauet *et al.*, 2005).

Other capsid proteins

The location of polypeptide IIIa (570 aa for Ad2) in the capsid has recently been defined by difference mapping to a position below the penton base (Saban *et al.*, 2006). The N-terminal region is highly helical and as well as binding to the penton base is also associated with hexons and protein VI. Some evidence of binding to core proteins V and VII (see below) has also been reported (Boudin *et al.*, 1980). There appears to be 60 monomers per virion (Rux & Burnett, 2004), in agreement with the finding of five helical clusters interacting with the other capsomeres at the apex of the capsid (Figs 1 and 2b, c).

Polypeptide VI (500 aa for Ad2) has two long α-helices and one of these binds to hexon (Matthews & Russell, 1994, 1995) and can be discerned within a cavity at the base of the hexon (Saban *et al.*, 2006). However, a number of reports examining the stoichiometry of VI (Chelius *et al.*, 2002; Lehmberg *et al.*, 1999) imply that there are only about 360 copies per virion. Since VI also associates with IIIa at the apices, it is not clear if there are multimers of VI or if there is only partial occupancy of the hexon cavity. It has also been suggested that VI is located underneath the peripentonal hexons as trimers of dimers (Stewart *et al.*, 1993), and this has been assumed in formulating Figs 1 and 2 (b, c).

The location of polypeptide VIII (140 aa for Ad2) has been difficult to resolve, but there is now agreement that it can be located to the inner side of the capsid in two non-equivalent positions: viz. five copies in a ring around the peripentonal hexons connecting them to the GONs and three copies in further rings around the threefold axes presumably stabilizing the GONs, giving a copy number of 120 (Fig. 2b). In looking at the internal structure at the apex of the virion (Fig. 2c) it will be noted that VIII provides a bond between the peripentonal hexons and the rest of the capsid. It is possible that this is a relatively weak link which can be ruptured when the penton base is detached during virus entry into the cell, allowing the H1 hexons to be released, leaving a hexon shell (see below).

Polypeptide IX (140 aa for Ad2) has recently been shown to have a great propensity to form coiled coils and it has been proposed that its N terminus is situated at the middle of each facet, while the C terminus forms a four helix bundle, with one helix interacting with the hexon HVR4 loop thus assigning it externally to the adjoining facet edges, in agreement with its copy number of 240 (Marsh *et al.*, 2006; Saban *et al.*, 2006). However, there are indications that the C termini could be more flexible in their locations and there is some disagreement about these assignments (Marsh *et al.*, 2006) (Fig. 2a).

The remaining structural polypeptides are associated with the virus core, i.e. the virus genome and the core polypeptides V, VII, Mu as well as terminal protein (TP). Two other polypeptides, IVa2 and the protease, could also be considered as components of the core (see below). The cores can be visualized by negative staining electron microscopy, but only as rather diffuse entities (Fig. 4c).

There is very little structural information regarding polypeptide V (368 aa for Ad2); it seems to be associated loosely with polypeptide VII and the virus DNA (Harpst *et al.*, 1977; Russell *et al.*, 1971) and tighter with polypeptide VI (Matthews & Russell, 1998b), thus providing a bridge between the core and the capsid. Since there are about 160 copies of V and 360 of VI (Chelius *et al.*, 2002; Rux & Burnett, 2004), there may be multimers of VI involved in the binding to polypeptide V (see above).

Polypeptide VII (174 aa for Ad2) is highly basic and binds tightly to DNA (Russell & Precious, 1982) and since there are over 800 copies per virion these appear to be spread along the length of the virus DNA, although there is some indication of the formation of particulate structures and supercoiling on treatment with nucleases (Goding & Russell, 1983b; Nermut *et al.*, 1975; Wong & Hsu, 1989). Another very basic component of the nucleoprotein core is Mu (36 aa), with about 100 copies per virion. Mu has

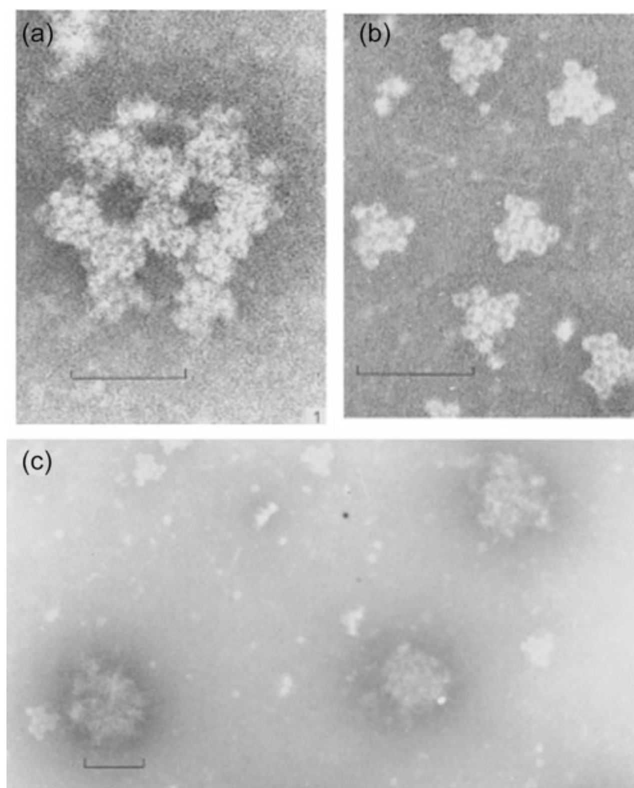

Fig. 4. Hexon shells and cores. On heating adenovirus hexon (a) shells (b) and cores (c) can be viewed by negative staining. The bar represents 500 Å (50 nm; Russell *et al.*, 1967b).

properties akin to those found in protamines. Nothing is known of its disposition along the DNA.

A protein which is also found in the virion is polypeptide IVa2 (449 aa for Ad2), present as only a few copies. This protein binds to DNA (Russell & Precious, 1982) at a specific stretch of the virus DNA and is critical to the packaging process (see below) and seems to be present as a multimer (Tyler *et al.*, 2007). The terminal protein (653 aa for Ad2) is covalently attached to the 5′ termini of the virus DNA (Rekosh *et al.*, 1977); however, it is not known if this interacts with other structural proteins. A critical structural protein is the virus protease (adenain) (204 aa for Ad2): it is necessary to produce the infectious virus particle from the procapsid by cleaving the precursors to the structural proteins IIIa, VI, VII, VIII, as well as pTP and precursor of Mu (polypeptide X) (Anderson, 1990; Mangel *et al.*, 2003; Weber, 1976; Webster *et al.*, 1989). Crystal structure determination has shown that the molecule is folded into two domains that are similar to that of the cysteine protease papain (McGrath *et al.*, 2003). There is some disagreement as to the number of copies per virion – varying from 10 to 70. In Fig. 1 it is surmised that there are 12 copies and that these are associated with the vertices of the icosahedron. The protease is activated by binding (non-specifically) to virus DNA, but requires binding of the C-terminal peptide from VI for optimal activity (McGrath *et al.*, 2003) (see below). Hereafter, the prefix p refers to the precursor of the cleaved polypeptide.

This review seeks to examine the role of these structural components in determining the course of infection. In doing so it will become evident that the capsid is not just a shell, but a vehicle for delivering specific functions which will decide the fate of the virus–host interaction.

Initial events in infection

The fibre is the first virus component to interact with a given tissue. In a natural infection this will probably occur via an aerosol into either the respiratory or gastrointestinal tracts, the oropharynx or conjunctiva. In all cases, there may very well be physical barriers to entry and it has been shown in mouse model systems that the glycocalyx and tethered mucins can inhibit adsorption of virus to the lung tissue (Stonebraker *et al.*, 2004). However, by extensive analysis of infection *in vitro* it has become evident that there are a number of binding receptors (Zhang & Bergelson, 2005) on a variety of cells viz. the major receptor for most adenoviruses is the so-called CAR receptor (coxsackie adenovirus receptor), which is a member of the immunoglobulin superfamily and is involved *in vivo* in the formation of tight junctions (Coyne & Bergelson, 2005, 2006; Philipson & Pettersson, 2004) such as in polarized epithelial cells. In general terms, viruses in species A, C, E and F interact with CAR receptors while with species D and E other receptors are utilized.

Most of the species B viruses bind to a ubiquitously expressed membrane complement regulatory molecule CD46 (Gaggar *et al.*, 2005; Marttila *et al.*, 2005; Segerman *et al.*, 2003b; Sirena *et al.*, 2004), but the species B viruses Ad3 and Ad7 bind to a related molecule CD80 or CD86 (Marttila *et al.*, 2005; Short *et al.*, 2004). Members of the group D viruses can also utilize ubiquitous sialic acid receptors (Arnberg *et al.*, 2002; Segerman *et al.*, 2003a). There are also reports of heparin sulphate glycosoaminoglycans (HSGAGs) binding Ad2 and Ad5 viruses (Dechecchi *et al.*, 2001) (Fig. 3).

Attachment to these initial receptors normally occurs in concert with the binding of the RGD peptide on the penton base to cellular $\alpha v\beta 3/\alpha v\beta 5$ integrins (Mathias *et al.*, 1994), thus facilitating virus internalization via clathrin-coated vesicles (Patterson & Russell, 1983) and into endosomes for further processing. This interaction with integrins induces a variety of cellular responses, e.g. activation of PI3 kinase (Li *et al.*, 1998b) and Rho GTPases (Li *et al.*, 1998a), both of which are important in altering the cytoskeleton in order to facilitate internalization. The later stages of virus trafficking into the cell can vary depending on the portal of entry and the serotype (Stone *et al.*, 2007a). However, it may be that *in vivo* other factors are important, e.g. targeting of Ad5 to the liver requires the involvement of coagulation factors and hexon (Waddington *et al.*, 2007, 2008).

The structural components can therefore play a critical part in facilitating efficient transport into the nucleus as well as activating the cell to respond to infection. As a first step to understanding these events, a more detailed assessment of the functions of the various structural components of the virion is made below.

Functions associated with structural components

Fibre

The fibre consists of a rod and a knob attached to the penton base as described above. The length and flexibility of the rod component can vary quite considerably among serotypes. The length is a function of the number of pseudorepeats present and can range from 3 to 23, providing very short fibres for serotype 3 and long fibres for serotype 12. A single repeat (as noted above) is characterized by a consensus sequence coding for two anti-parallel β-strands connected to a β-turn and a surface-exposed loop of variable length. Insertions of two to four residues in the β-turn at given positions can create hinge regions, which provide sufficient flexibility to the fibre rod to allow binding to two separate sites (such as CAR and integrins) and contributes significantly to the ability of the virus to bind to and infect cells (Lecollinet *et al.*, 2006; Nicklin *et al.*, 2005; Wu *et al.*, 2003). As noted above, the fibre knob has a number of exposed loops on the sides and top, and these expose variable sequences, providing the range of receptor binding sites characteristic of the different species (Fig. 3c, d). The characteristics of the fibre structure are reasonably well conserved within the species and contribute significantly to the tissue tropism and to the disease patterns observed. On binding it seems as if considerable structural alterations of the receptor

occurs (Persson *et al.*, 2007). In addition, the haemagglutinating properties of species are a function of the variable sequences on the fibre knob, and these can in turn be used to distinguish species A to F adenovirus isolates by a single-step PCR technique (Pehler-Harrington *et al.*, 2004) and provide an indicator of disease potential (Russell, 2000). The ability of the virus to bind to different cells is obviously a key feature of the infection process and governs the potential pathogenicity of the virus.

Species A adenoviruses consists of three serotypes, 12, 18 and 31, which utilize CAR and RGD binding to integrins and have been grouped thus because they readily produce tumours in hamsters. They also show incomplete haemagglutination with rat erythrocytes and have a fibre length of approximately 20 β-repeats – properties which they share with members of the C species. However, there are significant differences in the prevalence and disease characteristics of these two species – C being associated with extensive mild respiratory disease while A is relatively rarely isolated from humans. Detailed studies on the knobs of adenoviruses 2 and 12 have shown that, although the location of the CAR-binding sites are similar, there are large differences in the charge distribution, accounting for up to 100-fold differences in binding affinities for soluble CAR. This would be consistent with differing propensities of these species to infect respiratory epithelia (Howitt *et al.*, 2003). It may also be that species A fibres could bind more effectively to a receptor not yet recognized.

Species B adenoviruses can be subdivided into B1 (Ads 3, 7, 16, 21 and 50) and B2 (Ads 11, 14, 34 and 35) (Benko *et al.*, 2000). Most of them cause respiratory and/or ocular infections. Three B2 viruses have, in addition, been associated with renal infections and severe disease in immunocompromised patients. There have been rather conflicting results in terms of receptors for species B viruses, but it has been claimed that all bind CD46 (Fleischli *et al.*, 2007; Marttila *et al.*, 2005). Ads 3, 7 and 11 also bind the related receptors CD80 and CD86, and it has been claimed that these receptors can also be utilized by all species B adenoviruses (Short *et al.*, 2006). However, contrary results for Ad 3 have been demonstrated, suggesting that there may be receptors other than CD46, CD80 and CD86 (K. Hall and G. E. Blair, personal communication).

Adenoviruses 2 and 5, members of species C, are the most studied and the availability and characteristics of their cell receptor CAR on target tissues have been analysed extensively. CAR is a member of the immunoglobulin family with two extracellular domains that is present in intracellular junctions, such as the cardiac intercalated disc and the tight junctions of polarized epithelial cells. Its tissue distribution in humans is not well defined, but its mRNA seems to be present in a range of organs such as the heart, brain, pancreas, intestine, lung, liver and kidney (Zhang & Bergelson, 2005). Nevertheless, CAR expression does not seem to follow virus tropism *in vivo* and it seems likely that there are anatomical and other immunological barriers to infection (Fechner *et al.*, 1999). To some extent, this can be explained by the fact that CAR is on the basolateral membrane, below the level of the tight junction which forms a barrier regulating the transport of water and molecules into the cell. Infection initially appears to be very limited until virus and soluble fibre is released from infected cells and compete with the CAR and mediating cell adhesion. The epithelial layer is then permeabilized, allowing more efficient infection (Walters *et al.*, 2002). A recent study indicates that lactoferrin, a component of tear fluid, may have a role in acting as a bridge for CAR-independent binding of virus to epithelial cells (Johansson *et al.*, 2007). Another factor which limits infection is the apparent requirement for the virus to bind to two receptors, e.g. CAR and integrins via the penton base (see below). This will impose geometric constraints depending on the surface distribution of the two receptors. Thus, a longer more flexible fibre may be advantageous to allow infection.

Although the above pathways have been reasonably well delineated, the fact that there is sufficient variability in the fibre structure allows binding to other receptors. Thus, it has been established that HSGAGs (heparin sulphate glycosoaminoglycans) can mediate CAR-independent attachment and infection by species C viruses (Dechecchi *et al.*, 2001). It has been hypothesized that a basic sequence on the proximal fibre shaft, KKTK, is responsible for the binding (Smith *et al.*, 2003). Interestingly, this motif is present on all species C viruses, but absent from all other adenoviruses that have been sequenced (Zhang & Bergelson, 2005) (Fig. 3d). Since HSGAGs are prevalent in the extracellular matrix and the cellular glycocalyx, these may also act as virus chelators inhibiting infection. A systematic approach to identify cellular proteins binding to fibre knobs has indicated that there are other knob ligands, e.g. blood proteins which probably play an important role *in vivo* (Gaggar *et al.*, 2007). Indeed, liver tropism hampers systemic administration of adenovirus vectors, and it has been shown that ablation of the HSGAG receptors on the fibre can lead to abrogation of liver binding (Bayo-Puxan *et al.*, 2006). The fibre knob also seems to be important in fibre protein synthesis and encapsidation (Henning *et al.*, 2006).

As noted above, classification is partially based on agglutination of red blood cells and this is fibre-mediated (Eiz & Pring-Akerblom, 1997) via a so-called gamma determinant located on the loops on the knob (Nicklin *et al.*, 2005). Recent studies have indicated that sequestration of virus via the fibre on blood components such as coagulation factor IX, complement, C4-binding protein and platelets as well as Kuppfer cells in liver sinusoids play a significant role in defining the route and outcome of virus infection (Baker *et al.*, 2007; Nicklin *et al.*, 2005; Shayakhmetov *et al.*, 2004, 2005b; Stone *et al.*, 2007b).

Species D adenoviruses, although containing 32 different serotypes, are rarely isolated from humans. The major exception to this occurs with serotypes 8, 19a and 37,

which cause severe epidemic keratoconjunctivitis. It is also significant that the prototype isolate of Ad19 (Ad19p) does not cause disease. There appears to be two mutations on the top of the fibre knob of 19p which lead to a partial loss of the high positive charge, but it has been difficult to relate these changes to altered tropism and cell binding (Burmeister *et al.*, 2004). With several species D viruses it is clear that sialic acid functions as a receptor via a charge-dependent interaction (Arnberg *et al.*, 2002), and that binding takes place at the top of the fibre trimer (Burmeister *et al.*, 2004) (Fig. 3d).

There is only one member of species E adenovirus, adenovirus type 4, and this appears to have some genetic input from both species C and B viruses (Gruber *et al.*, 1993) and to be responsible for rather more clinically severe diseases, both respiratory and pharangoconjuctival. As yet, there has been no definitive studies on receptor usage.

Human enteric adenoviruses of species F have a striking difference from the other species in that they have two lengths of fibre in the same virion in about equal numbers (Kidd *et al.*, 1993; Favier *et al.*, 2002). The longer fibre has been shown to bind CAR, but the binding site of the short one has not yet been defined. The short fibre head has a number of differences from the standard knob, particularly in terms of sensitivity to pepsin, and does not bind CAR (Seiradake & Cusack, 2005).

Recent crystallographic studies have shown that, while the essential fibre structure is retained among the human serotypes, there are significant differences in the electropotentials on the surfaces of the fibre heads: thus the isoelectric potentials can vary from 4.97 (Ad3) to 9.4 (Ad37) (Arnberg *et al.*, 2002). There are also critical changes in the loops emanating from the fibre knobs (Fig. 3c), e.g. the AB loop of Ad3 does not allow CAR binding as a result of changes in amino acid residues (Durmort *et al.*, 2001).

Penton base

As noted above, the penton base occupies a strategic position at the apices of the icosahedral capsid and can play a key role in stabilizing the capsid by interacting with the neighbouring capsomeres, peripentonal hexons and IIIa. However, it is well established that the penton base is the weakest site in the capsid, being sensitive to heat, trypsin, pH and to changes in ionic strength (Rexroad *et al.*, 2006a, b; Russell *et al.*, 1967b; Wiethoff *et al.*, 2005). As indicated above, the RGD sequence in the variable loop of the penton base (Fig. 3b) seems to be essential in co-operation with the fibre knob in attaching and internalizing the invading virion via clathrin-coated pits. This dual binding depends on the flexibility of the fibre in binding to the cell receptor and the base in reacting with cellular integrins (Fig. 5b). The RGD loop also seems to play a role in facilitating the release of the virion from the endosome (Shayakhmetov *et al.*, 2005a). Moreover, it has been reported that fibres can detach themselves from the penton base following the reaction of the penton with integrins (Nakano *et al.*, 2000). Thus, if this is the case, it seems likely that the penton base, perhaps in association with IIIa and/or VI, can be regarded as the main facilitator for virion entry and penetration into the cell (see below). Moreover, it is interesting to note that pentons can aggregate readily to form dodecahedra which can interact with cellular components in a different way from virions (Fender *et al.*, 2005, 2008; Fuschiotti *et al.*, 2006; Vives *et al.*, 2004). Isolated pentons have also been shown to interact with cells and follow a different pathway (Rentsendorj *et al.*, 2006). In view of these apparently discordant observations, it is not clear what role the pentons play at the post-entry stages *in vivo*, e.g. it has been shown that they can interact with a number of cellular components such as ubiquitin ligases (Chroboczek *et al.*, 2003). Neutralizing antibodies against the penton base have been demonstrated in patients' sera and the responsible epitopes have been mapped proximal to the RGD sequences, confirming the importance of the RGD recognition step in infection (Hong *et al.*, 2003). A very old finding that isolated penton base can very rapidly lead to cell rounding *in vitro* (Pereira, 1958; Russell *et al.*, 1967a) could also be explained by the RGD interaction, although some other highly conserved motifs appear to be essential for this 'early cytopathogenic' effect (Madisch *et al.*, 2007): it may be that other properties of the penton base have yet to be defined. It is also evident that the pathways of entry may differ according to cell type as well as with different species of adenovirus. Thus, infection of lacrimal acinar cells by adenovirus type 5 does not require the participation of penton base, and they seem to remain on the cell surface after infection (Xie *et al.*, 2006).

Hexon

The hexon, being the major capsid component, is one of the principal players in establishing the immune responses – humoral, cellular and innate (see below). The overall stability of the virus structure during infection depends on the interactions of hexons between themselves and with the other structural components. The peripentonal hexons in association with the penton base, VI and IIIa seem to be less stable in the initial stages of infection, perhaps by being more susceptible to pH changes occurring in the endosome, thereby allowing access to the virion core. It may be that the 'cement' polypeptides IX on the outer facet provide better hexon-hexon bonding than that with polypeptide VIII (see below). On heating purified virus it was noted earlier (Russell *et al.*, 1967b) that, as well as GONs, a shell of hexons composed of 20 GONs (perhaps associated with polypeptides VI and VIII) could be discerned (Fig. 4a), and it may be that this structure is the one that can persist in the acidic environment of the endosome (see below) and is sufficiently stable to protect the virus genome on its journey to the nucleus. The hexon also seems to have a significant role in infection of hepatocytes by binding tightly to coagulation factor X (Kalyuzhniy *et al.*, 2008).

Polypeptide IIIa

As noted above, IIIa has a highly helical structure with at least 14 helices at the N-terminal two-thirds and two at the C-terminal third (Saban *et al.*, 2006). Structural studies place the 60 copies at the 12 apices with five copies in close proximity to the underside of the penton base. The N termini seem to mediate the interaction with the penton base (Saban *et al.*, 2005). This polypeptide appears to be highly phosphorylated (Russell & Blair, 1977) at multiple sites as determined by two-dimensional polyacrylamide electrophoresis (Russell & Kemp, 1995). Furthermore, a motif analysis indicates that there are a number of putative protein kinase sites towards the C terminus (unpublished observations). Phosphorylation of IIIa occurs early in infection (Tsuzuki & Luftig, 1983), and in keeping with these findings, it has been shown that the virus contains protein kinase activity (Blair & Russell, 1978; Tsuzuki & Luftig, 1985a). Since IIIa appears to associate with the other proteins at the vertex, it could be that activation of a putative virion kinase and phosphorylation of IIIa is an important step in the initial stages of virus disassembly. However, there have as yet been no characterization of a kinase in terms of structural components (Fig. 5c).

Polypeptide VI

The structure of VI and its precise location in the virion is unclear but, as noted above, it seems to be in contact with the inner cavity of hexons, possibly as a trimer of dimers (Stewart *et al.*, 1993), and also associated with IIIa and penton base at the vertex as well as the core protein V (Chatterjee *et al.*, 1985; Matthews & Russell, 1998b). This being the case, it seems likely that the hexon association could be with the peripentonal hexons (Fig. 2c). The current evidence indicates that VI is released from the virion when the acidic environment of the endosome triggers a conformational change, and can then mediate disruption of the endosomal membrane to allow the transport of the virus towards the nucleus (Wiethoff *et al.*, 2005). There is also a role for VI in virus maturation, since cleavage of newly synthesized pVI by the virion protease and release of the C-terminal peptide leads to activation of the protease (see below) (Honkavuori *et al.* 2004; Mangel *et al.*, 1993; Webster *et al.*, 1993; Webster & Kemp, 1993). Prior to cleavage, pVI facilitates the import of newly translated hexon into the nucleus, and after cleavage it participates in virion assembly (Wodrich *et al.*, 2003).

Polypeptide VIII

Very little is known about the function of this protein, although its location suggests that it may have a role to play in stabilizing the GONs (see above); this is borne out by reports that a mutation in VIII induces thermolability in the virion (Liu *et al.*, 1985). It is interesting that component p30 of bacteriophage PRD (which shows some structural similarities to adenovirus) shares properties with VIII and seems to play a role in binding the icosahedral facets of the virus (Abrescia *et al.*, 2004). In bovine adenovirus 3, polypeptide VIII has been shown by yeast two-hybrid analysis to bind to polypeptide IVa2 (Singh *et al.*, 2005), but it is not clear whether this interaction modifies the function of IVa2 (see below).

Polypeptide IX

As noted above, this component seems to act as a stabilizer of the capsid, being situated on the external facets. However, polypeptide IX only seems to be present in the family *Mastadenoviridae*, and indeed mutant HAd 5 viruses lacking IX can be propagated, although they are more heat-sensitive than wild-type HAd5 (Boulanger *et al.*, 1979; Colby & Shenk, 1981). The N terminus is situated centrally on the external facet of nine hexons or GONs (Fig. 2a) and is preserved between serotypes, and seems to confer thermostability. The central alanine-rich region and the C-terminal coiled-coil regions appear to be more freely available and can be used as a basis for anchoring other polypeptides to the capsid, and are therefore useful in constructing gene vectors (Vellinga *et al.*, 2004) and for tagging the virus with a fluorescent marker to facilitate tracking of virus in infected cells (Meulenbroek *et al.*, 2004). A recent report shows that a triple mosaic of IX can be constructed with three different modifications at the C termini (Tang *et al.*, 2008).

Core proteins V, VII, Mu, protease and TP

The core proteins V and VII interact with the virus DNA in such a way that the virus template is available at least for limited *in vitro* replication (Goding & Russell, 1983a), and since protein V seems to be dispensable (Ugai *et al.*, 2007), and it is not present in the family *Atadenoviridae* (Gorman *et al.*, 2005), it is plausible to postulate that virus DNA plus protein VII may be the functional template for replication and transcription *in vivo*, at least for the initial stages (Chatterjee *et al.*, 1986; Haruki *et al.*, 2006; Spector, 2007). Following endocytosis, and disruption of the endosome, entry of the virus core into the nucleus is governed by multiple signals in VII which target the genome to the nucleus and to the nucleolus (Lee *et al.*, 2003; Wodrich *et al.*, 2006). Transport to the nucleus seems to be via the microtubule network and association with the microtubule organizing centre (MTOC). A number of cellular proteins have been implicated in the import into the nucleus, i.e. importins, histone H1, hsp70 and nuclear export factor CRM1 (Saphire *et al.*, 2000; Strunze *et al.*, 2005; Trotman *et al.*, 2001). Since it has been shown by yeast two-hybrid analysis that protein V has an affinity with a cellular protein, p32, which shuttles between the nucleus and mitochondria, it may be that there are a number of routes which the invading virus template can hijack to reach the nucleus (Matthews & Russell, 1998a). A recent paper suggests that protein VII is the major mediator of virus DNA import into the nucleus, facilitated primarily by

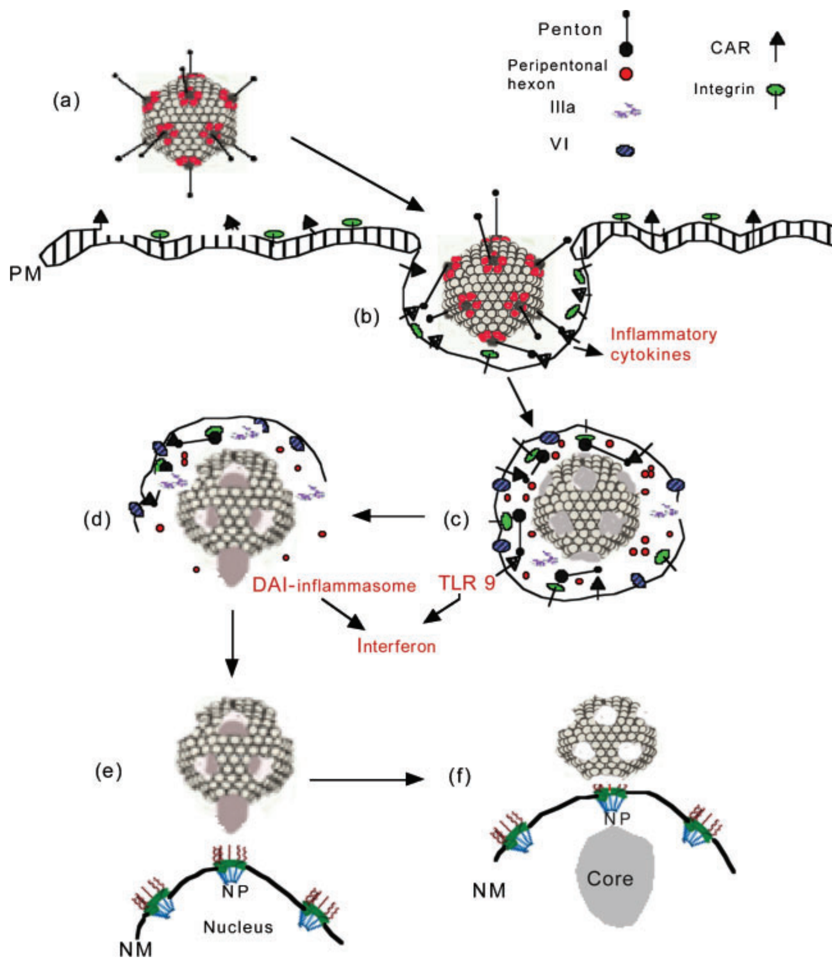

Fig. 5. Early events in infection. (a) Adenovirus at the plasma membrane (PM), peripentonal hexons are in red. (b) Binding of pentons to integrins and receptors followed by phagocytosis. (c) A phagocytic vesicle (endosome/lysosome) and disruption of the adenovirus particle releasing peripentonal hexons, IIIa and VI and revealing the core. (d) Rupture of endosome/lysosome and release into cytoplasm of hexon shell with a metastable core (virus DNA with TP, VII, V, Mu and possibly protease). (e) Hexon shell at nuclear pore (NP) with core being released into the nucleus. (f) Core in the nucleus targeting cellular chromatin. Key steps in the induction of the immune response are noted in red. NM, nuclear membrane.

transportin (Hindley *et al.*, 2007). There is also evidence that, prior to nuclear import, V and VII interact with the Golgi apparatus via the MTOC and then travel to the endoplasmic reticulum prior to nuclear import (C. Hindley and D. A. Matthews, personal communication). After import, cellular proteins SET and pp32 (components of two multiprotein complexes of cellular chromatin) also play a role in altering the template properties (Xue *et al.*, 2005) and a template-activating factor (TAF-1) binds to VII, leading to remodelling of the virus chromatin (Gyurcsik *et al.*, 2006; Haruki *et al.*, 2006). Protein VII seems to dampen early transcription, but eventually transcription of E1A leads to the release of VII, followed by remodelling of the virus chromatin and late transcription (Chen *et al.*, 2007; Johnson *et al.*, 2004).

pVII, in addition, participates in virus assembly by interacting with protein IVa2 and L1 52/55K, which are both bound to the specific packaging sequences on the virus DNA (Zhang & Arcos, 2005). It is interesting that protein V, at least in Ad5 infected cells, seems to home to both the nucleus and the nucleolus (Matthews & Russell, 1998b) and induces redistribution of nucleolin and B23 from nucleolus to cytoplasm (Matthews, 2001). B23, an acidic protein and a component of template-activating factor 3, seems to interact with both V and pre VII and could facilitate dissociation of core proteins (Samad *et al.*, 2007), and thereby reveal the virus template for replication and/or transcription (see above). Furthermore, a nucleolar component 'upstream binding factor' appears to be involved in virus DNA replication (Lawrence *et al.*, 2006), but it is not clear whether these effects are directly dependent on protein V. It may be that V facilitates these nucleolar functions without being absolutely necessary. Another possibility is that the small core protein Mu can duplicate these nucleolar functions of V (Ugai *et al.*, 2007). This small peptide can exclusively target the nucleolus (Lee *et al.*, 2004), and appears to play a part as a precursor (preMu) in modulating expression of E2 early proteins, resulting in a shift in late protein expression. PreMu is formed as a precursor molecule, polypeptide X, which presumably condenses the virus prechromatin by virtue of its two basic domains, and following cleavage by the virion protease there is a conformational change to facilitate packaging of the core complex.

There seems to be efficient binding between V, VII and Mu (Chatterjee *et al.*, 1985) to form a complex, although the precise topology of the complex with the virus DNA is

unknown. The nucleosome-like structures could be formed by six molecules of VII and linked by V proteins (Sung *et al.*, 1983). It is plausible that some modification of these protamine-like proteins, such as phosphorylation (Blair & Russell, 1978; Tsuzuki & Luftig, 1985b) or acetylation, could also be involved in alteration of the virus template for transcription and/or virus DNA replication.

The adenovirus protease is an essential feature of the virion and is synthesized in an inactive form that is then activated in two stages: it first binds non-specifically to virus DNA (Gupta *et al.*, 2004) and becomes partially activated to cleave the precursor to polypeptide VI, which presumably is proximal to the protease. The C-terminal 11 aa peptide (pVIc) which is produced binds to the protease and bridges its two domains (see above) to induce its full activation. It has been postulated (Mangel *et al.*, 2003) that the protease–peptide complex then moves along the viral DNA, cleaving the other virion precursor proteins (IIIa, VII, VIII, IX, TP and X-precursor to Mu). Indeed all of these polypeptides are either associated with the virus DNA or proximal to polypeptides which are (e.g. IIIa and VII). Interestingly an early report (Chatterjee & Flint, 1987) suggested that the protease may be phosphorylated and this may have some role in its function within the virion. Another function of the protease, which facilitates cell lysis at the early stage of infection, is its ability to cleave both cytokeratin and actin – indeed the C terminus of actin is homologous to that of pVIc (Brown & Mangel, 2004; Gupta *et al.*, 2004; Mangel *et al.*, 2003). The importance of the protease is underlined by the finding that a protease defective *ts* mutant cannot proceed beyond the initial stages of infection (Russell & Kemp, 1995; Weber, 1976). Following cleavage, there seems to be significant conformational changes to the structural proteins, e.g. the cleaved C-terminal peptide of polypeptide pVI can then facilitate capsid assembly (Wodrich *et al.*, 2003) and the preMu polypeptide seems to have a role in the regulation of early proteins – a property not apparent with the matured polypeptide (Lee *et al.*, 2004).

The structural protein IVa2, which is present only as a few molecules attached to the virus DNA at the so-called A repeat sequences at the left-hand end and to the major late promoter, plays a major part in both encapsidation and regulation of late transcription. Packaging of the virus DNA involves specific binding of IVa2 to virus DNA as a multimeric complex with a virus-coded non-structural protein L1 52/55K and with pVII, which is bound non-specifically to the virus DNA (Ostapchuk *et al.*, 2005; Perez-Romero *et al.*, 2005; Tyler *et al.*, 2007; Zhang & Arcos, 2005). Another non-structural protein, L4 22K, also appears to be involved in packaging along with IVa2 (Ewing *et al.*, 2007; Ostapchuk *et al.*, 2006).

The transcriptional complex that regulates the major late promoter (MLP) is claimed to be made up of a dimer of IVa2 and the non-structural protein, L4 33K (Ali *et al.*, 2007; Pardo-Mateos & Young, 2004). Given that there are only a few molecules of IVa2, specific binding of IVa2 to virus DNA towards the left-hand end of the conventional virus genome and also to the MLP (nearer the middle) could be accommodated if the core complex is folded so that the A sequences and the MLP promoter are proximal (e.g. as in Fig. 1). However, recent studies suggest that IVa2 occurs at a single vertex in the mature virion (Christensen *et al.*, 2008) and consistent with its packaging function it binds ATP (Ostapchuk & Hearing, 2008). To add further complexity, it has also been reported by yeast two-hybrid analysis that pVIII binds to IVa2 in a porcine adenovirus (Singh *et al.*, 2005).

A further structural component is terminal protein (TP), which is covalently attached to the virus DNA termini (Rekosh *et al.*, 1977) and may facilitate circularization of the virus genome (Ruben *et al.*, 1983). The terminal protein is made as a precursor pTP, with cleavage by the virus protease at two sites being essential for virus replication (Webster *et al.*, 1997b). pTP, along with the virus-coded polymerase and cellular protein NF1, primes virus DNA replication (Liu *et al.* 2003; Hay *et al.* 1995; Webster *et al.*, 1997a). pTP also plays a role in binding to the trifunctional enzyme CAD (carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase) in the nuclear matrix, and thereby anchors the replication complex to this site (Angeletti & Engler, 1998).

Host response to adenovirus infection

Adenoviruses have been utilized extensively as gene therapy vectors but, although they can package and deliver foreign genes to appropriate cells, the host response is so effective that, in most cases, the transduced cells are rapidly eliminated, often accompanied by considerable toxicity. Both arms of the immune system to adenoviruses, i.e. innate and adaptive, are involved (Burgert *et al.*, 2002). The virus structural components are key instigators of these responses.

Induction of innate immunity

On infection by adenoviruses rapid innate responses are induced as the host seeks to repel the invader. This innate early response occurs as a result of the interaction of the virus with the cell and does not necessarily depend on the transcription of any virus genes. The nature of the innate response network triggered varies significantly depending on receptor usage and cell type, yielding a complex signalling cascade with diverse outcomes. Many of these pathways eventually lead to the upregulation of the transcription factor NF-κB and interferon regulatory factor 3 (IRF3) and the production of interferon (Randall & Goodbourn, 2008). Interferon can also be induced after transcription of early genes (Russell, 2000).

The earliest reports of interferon induction mediated by adenovirus infection were performed in chick embryo fibroblasts (Béládi & Pusztai, 1967; Ho & Kohler, 1967). Intriguingly, interferon induction was observed in the absence of virus-encoded protein synthesis, and was only

partly ablated if the virus was heated or UV-inactivated, thus implicating virus structural components as the precipitating components. The infection of chick cells was abortive and further investigation identified two *ts* mutants that failed to stimulate interferon induction (Ustacelebi & Williams, 1972). Analysis of these mutants suggested that the mutant virions were unstable and that, surprisingly, protein VI was possibly defective (Tarodi *et al.*, 1979). There have been no further investigations using this system.

Later studies have confirmed that interaction of the viral capsid with the host cell is sufficient to activate the pathways leading to inflammatory responses (Muruve, 2004). Thus, on infecting the human respiratory epithelial cell line A549 with Ad5, it has been demonstrated that fibre (and not hexon or penton base) was sufficient for the immediate pro-inflammatory response as measured by the induction of extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and the nuclear translocation of NF-κB. Moreover, the interaction of the fibre with the cellular CAR (and not with HSGAG) seems to have been the critical initial event (Tamanini *et al.*, 2006). Other authors have come to different conclusions regarding the initial events, e.g. Liu *et al.* (2003) noted that RGD motifs in the penton base were critical for induction in endothelial cells. Recently, an in-depth transcriptome analysis using a mouse cell line permissive for Ad5, as well as mouse liver cells, revealed early dysregulation of multiple cellular genes with both infectious virus and with UV-inactivated virus (Hartman *et al.*, 2007a, b). There was a widespread alteration of gene expression even with empty capsids (Stilwell & Samulski, 2004), accompanied by a strong induction of MAP kinase and Jak/Stat pathways, all leading to inflammatory outcomes. In addition, there was activation of focal adhesion and tight junction functions, thus facilitating the further progress of the infection. Significant alterations in cellular gene expression were evident both early and late in infection (Granberg *et al.*, 2005; Granberg *et al.*, 2006; Miller *et al.*, 2007). During adenovirus infections, pathogen-associated molecular patterns (PAMPs) are detected by a limited number of cellular sensors, which come into play depending on the route of entry and the type of cell. Interestingly, toll-like receptors (TLRs) are significantly upregulated in the transcriptome analyses described above. There at least 13 TLRs in mammals and they have unique specificities (Randall & Goodbourn, 2008). TLR9 and the adaptor molecule MyD88 have also been implicated in infection of murine and human cells by Ad5 vectors (Yamaguchi *et al.*, 2007; Cerullo *et al.*, 2007). Peripheral blood mononuclear cells are rich in CD46 and on infection with adenoviruses belonging to species B and Ad37 induce efficient interferon production via plasmacytoid dendritic cells (pDCs; Iacobelli-Martinez & Nemerow, 2007). On the other hand, myeloid-derived murine dendritic cells produce interferon independent of TLR 9 on infection with Ad5 (Basner-Tschakarjan *et al.*, 2006). In contrast with species C adenoviruses, empty capsids of CD46-utilizing viruses did not induce interferon expression, and clearly the viral DNA in this case was required for the inflammatory response. Thus, there are differences in innate immune response pathways depending on the nature of the cells infected and on the adenovirus species (Fig. 5). After entry via the CAR receptor, species C viruses are released rapidly into the cytoplasm via early endosomes, whereas after entry via CD46, species B viruses associate with late endosomes and lysosomes. It is in the latter cellular compartment that TLR9 appears to concentrate (Kim *et al.*, 2008). TLR9 recognizes unmethylated CpG sequences in virus or microbial DNA, and it may be that the Ad genome is sufficiently different from the methylated CpG seen in normal cell DNA to signal that a foreign DNA is present. However, there are situations, as noted above, where the innate immune response is activated without the participation of TLR9. Here, the initial event seems to be the delivery of the virus genome into the cytoplasm and the interaction of the viral DNA with a cellular DNA-dependent activator (DAI; DLM-1/ZBP1) when released (Takaoka *et al.*, 2007). These routes are not mutually exclusive (Zhu *et al.*, 2007), and depend to a large extent on the nature of the cell being infected; thus pDCs, which are major inducers of type 1 interferons, operate mainly via TLR 9 and MyD88, whereas bone marrow-derived conventional DCs and lung fibroblasts may detect Ad DNA via a cytoplasmic DNA sensor (Nociari *et al.*, 2007). In both cases, a critical transcription factor for interferon induction, IRF3, is upregulated. There is now evidence that translational control via repressors is also an important factor in interferon induction (Colina *et al.*, 2008). Therefore, structural components of the virus, both capsid and DNA, play various roles in the induction of the innate immune response to adenovirus infection, and outcomes can vary depending on the virus species and the nature of the infected cell (Fig. 5). On considering the situation *in vivo*, it is clear that there are other factors, such as the complement system, which interact with these pathways (Kiang *et al.*, 2006). The induced innate immune response will obviously have an impact on the efficacy of gene therapy and application to vaccines (Hartman *et al.*, 2008; Muruve, 2004).

Adaptive immunity

The hexon, being the major capsid component, is a principal player in establishing the adaptive immune response – both humoral and cellular. As noted above, the hexon capsid can have at least nine hypervariable loops (Fig. 3a), and some of these appear to function as type-specific neutralizing antigens and thus define the serotype (Gall *et al.*, 1998; Madisch *et al.*, 2005; Pichla-Gollon *et al.*, 2006; Roberts *et al.*, 2006; Sumida *et al.*, 2005; Toogood *et al.*, 1992; Varghese *et al.*, 2004; Worgall *et al.*, 2005; Wu *et al.*, 2005). There have been a number of different conclusions about the number and specific locations of the neutralizing epitopes, perhaps reflecting the varying

experimental conditions used. However, it is interesting to see that a structural and phylogenetic analysis of all 51 human serotypes indicated that only three of these variable regions were sufficiently stable to withstand mutations without affecting the overall structure of the hexon (Ebner *et al.*, 2005; Rux *et al.*, 2003). One of the more recent studies (Pichla-Gollon *et al.*, 2006), using a chimpanzee adenovirus, has shown that conformational recognition of a single small loop on the hexon defines a major neutralization site by monoclonal antibodies, but this may not be applicable to human *in vivo* responses.

There are also a number of reports which point to the importance of both fibre and penton base in the induction of neutralizing antibodies (Hong *et al.*, 2003; Stallwood *et al.*, 2000). It has been shown that there is a synergy between the anti-capsid antibodies in contributing to neutralization (Gahery-Segard *et al.*, 1998). It seems likely that there are number of different mechanisms for virus neutralization, e.g. aggregation of virus may impede proper recognition at the cell surface, and there is also evidence that virus–antibody complexes can enter the cell and that inhibition occurs at a later stage (Varghese *et al.*, 2004).

Indeed, it has been demonstrated that an anti-hexon monoclonal antibody can block infection by arresting microtubule-dependent cytoplasmic transport (Smith *et al.*, 2008). In the context of type and species specificity, it is interesting to note that there is a correlation between the characterization of fibre and hexon – both in respect of haemagglutination and neutralization. Also, a recent study of 16 Ad C field isolates covering phylogenetic analyses of four serotypes indicated that the hexon and fibre proteins from different serotypes were incompatible – at least in nature (Lukashev *et al.*, 2008). It can be speculated that there may well be critical indirect structural communications between fibre and hexon, perhaps at the junction with the penton base at the apex which governs the overall stability of the virion. Another recent report has highlighted the importance of the cell's natural microbial defences (termed defensins) in combating adenovirus infection. These small peptides have been shown to inhibit the disruption of the virus by binding to the virus apex (Smith & Nemerow, 2008).

Cellular immune responses to adenoviruses have been reported both for cytotoxic $CD8^+$ T cells and for memory $CD4^+$ T cells (Leen *et al.*, 2004; Olive *et al.*, 2002; Onion *et al.*, 2007; Perreau & Kremer, 2005; Tang *et al.*, 2004, 2006). In the case of the $CD8^+$, HLA class I-restricted and multiple epitopes have been mapped to the highly conserved distal termini of the hexon. A recent extensive study (Leen *et al.*, 2008) examined adenovirus-reactive human cytotoxic T lymphocyte (CTL) lines with an overlapping hexon peptide library and detected 33 stimulating peptides with both HLA class I and II restriction. Of these peptides five were overlapping CD4 and CD8 T-cell epitopes. Moreover, all of these immunogenic peptides lay within the conserved region of the hexon. It has been noted that hexon can also be a potent adjuvant for activation of cellular immune responses (Molinier-Frenkel *et al.*, 2002). Only minimal responses to the other capsid proteins, fibre and penton base, were detected. Similar observations were made for the cell proliferative response (Flomenberg *et al.*, 1995), although there has been one report suggesting a role for fibre or IIIa (Souberbielle & Russell, 1995).

The role of natural killer (NK) cells in controlling Ad infections is not well characterised. Interestingly, depletion of the NK response in mice does not affect their sensitivity to murine Ad type (Welton *et al.*, 2008). With human Ad5, either expression of E1A or breakthrough expression from a E1 vector can stimulate cell surface expression of ligands for the powerful NK cell activity receptor NKG2D; however, this is countered by E3 19K (Routes *et al.*, 2005; Tomasec *et al.*, 2007; McSharry *et al.*, 2008). Remarkably, the Ad5 vector alone, even when inactivated by UV irradiation, stimulates a proliferation of NK cells in mice (Ruzek *et al.*, 2002).

It should be noted that adenoviruses also have very effective means of subverting the host immune responses by using the early E3 cassette of genes, but these are not discussed here (see review by Windheim *et al.*, 2004).

Epilogue

From the foregoing it has been seen that, although significant progress has been made in defining the structural characteristics of adenovirus, there are still many gaps in our knowledge of the complete functional significance of these components. It is obvious, as with other virus gene products, that the structural components have a range of properties – thus they do not merely provide a protective shell for the virus genome, but are essential in facilitating virus entry, survival and eventual successful replication in the face of the host's defence mechanisms.

Fig. 5 provides a simplified possible compendium for the early stages of virus infection. Assuming that the virus can gain access to a susceptible cell, and has overcome the physical and other barriers such as mucins (which can chelate the virus), then it has to find appropriate receptors before further progress can be made. This presumably will be a random process, facilitated by charge interactions of the fibre knob with the plasma membrane of the host cell. Moreover, there is a requirement for two receptors (e.g. CAR and integrin) to be contiguous (Fig. 5b). At least one of the receptors, in addition, will be variable depending on the virus species. The tissue distribution of the receptors will also be dependent on cell type (e.g. CAR is deficient in fibroblasts and in haematopoietic cells) and on tissue type (Nemerow, 2002). These early requirements play a large part in the subsequent fate of the infection in terms of pathology and disease outcome.

Given successful attachment to cellular integrins by the RGD motif on the penton base and to the fibre receptor, the next stage is triggering internalization of the virion via clathrin-coated pits and then endocytosis or macropinocytosis (Meier & Greber, 2004) (Fig. 5b). Once the virion is

enclosed within the endocytic vacuole (Fig. 5c) there is a profound change in the environmental pH, leading to its destabilization with the loss of capsomeres from the apices possibly with the concomitant formation of the hexon shell surrounding the virus core. In this situation, it seems likely that there will be release of VI and IIIa from the virion. It has been noted that protein VI can bind to the endosomal membrane, leading to its disruption (Wiethoff *et al.*, 2005) and perhaps acting in concert with the penton base (Fig. 5d). It is not clear if the penton is still intact at this stage, i.e. the fibres may have been removed at the early stages of initial binding (see above), but since the penton base does seem to be sensitive to proteolysis it may be degraded, especially if it is exposed to lysosomal enzymes. When released into the cytosol the genome is transported to the nuclear membrane, presumably by hijacking a cellular transport system (Leopold & Crystal, 2007). There is good evidence for the involvement of microtubules (Bailey *et al.*, 2003; Leopold *et al.*, 2000; Suomalainen *et al.*, 1999), but it may be that other mechanisms can be utilized, depending on the type of cell infected (Yea *et al.*, 2007). Most of these early stages of infection have been studied using species C adenoviruses and it is interesting that a recent report has indicated that Ad3, a member of species B, is dynamin-independent (Amstutz *et al.*, 2008), in contrast to Ad5. On reaching the nuclear membrane, the mechanism by which the virus genome is imported into the nuclear pore (Fig. 5e) has been shown to involve components of the nuclear pore complex (Greber *et al.*, 1997) and CRM1, a nuclear factor exported from the nucleus (Strunze *et al.*, 2005). In any case, it is probable that the hexons are mostly shed at the nuclear pore and the virus core enters the nucleus (Matthews & Russell, 1998b). Thereafter, the virus core proteins fulfil various functional roles in programming the nuclear machinery for transcription and replication (Russell & Matthews, 2003) in much the same way as the capsid proteins facilitated the early events in infection.

From the above, it is evident that all the structural components of the virus fulfil many functional roles in the infectious process, and in so doing activate the cellular and host defence mechanisms. The outcome in terms of the fate of the host depends ultimately on whether these defence mechanisms are effective in combating the wiles of the infecting virus.

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