Journal of General Virology (1999), **80**, 1519–1527. Printed in Great Britain

Molecular determinants of adenovirus serotype 5 fibre binding to its cellular receptor CAR

G. Santis,[1] V. Legrand,[2] S. S. Hong,[3] E. Davison,[1] I. Kirby,[1] J.-L. Imler,[2] R. W. Finberg,[4] J. M. Bergelson,[5] M. Mehtali[2] and P. Boulanger[3]

[1] Department of Respiratory Medicine & Allergy, 5th Floor Thomas Guy House, Guy's Hospital, St Thomas Street, London SE1 9RT, UK

[2] Department of Gene Therapy, Transgene SA, 11 rue de Molsheim, 67000 Strasbourg, France

[3] Laboratoire de Virologie et Pathogenese Moleculaire, CNRS UMR5812, Institut de Biologie, Faculté de Médecine, 34060 Montpellier, France

[4] Division of Infectious Diseases, Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA

[5] Division of Immunologic & Infectious Diseases, Abramson 1202E, Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Philadelphia, PA 19104, USA

Adenovirus (Ad) tropism is mediated in part through the fibre protein. The common coxsackie B virus and Ad receptor (CAR) was recently identified as the major receptor for subgroup C Ad serotype 5 (Ad5) and serotype 2 (Ad2) fibres. Effects of mutations in the Ad5 fibre gene were studied to assess domains of the fibre capsomer that could alter virus tropism without altering virus assembly and replication. All mutants that accumulated as fibre monomers failed to assemble with a penton base and proved lethal for Ad5 which suggests that the absence of infectious virions resulted in part from a defect in fibre penton base assembly. Cell binding capacity of all fibre mutants was investigated in cell binding competition experiments with adenovirions using CHO–CAR cells (CHO cells that have been transfected with CAR cDNA and express functional CAR). The results suggest that the R-sheet of the Ad5 fibre knob monomer contains binding motifs for CAR and that β-strands E and F, or a region close to them, may also be involved in receptor recognition.

Introduction

The first step in human adenovirus (Ad) infection consists of virus–cell recognition and attachment, involving the viral fibre protein and host cell surface receptor(s) (Defer *et al.*, 1990; Hennache & Boulanger, 1977; Svensson *et al.*, 1981). Internalization of virus particles is subsequently mediated through a specific interaction of the viral penton base protein with cell surface integrins (Bai *et al.*, 1994; Belin & Boulanger, 1994; Davison *et al.*, 1997; Wickham *et al.*, 1993, 1994). The fibre protein, which protrudes from the vertices of the icosahedral virion as a homotrimer, is divided into three domains: the tail, which binds to the penton base; the shaft, the length of which varies among various serotypes and is characterized by a repeating motif of approximately 15 residues (Green *et al.*, 1983; Signas *et al.*, 1985); and the knob, which is essential and sufficient for the binding of the adenovirion to host cells (Henry *et al.*, 1994; Xia *et al.*, 1994). Avian and human enteric adenoviruses Ad40 and Ad41 have two fibre genes (Davison *et al.*, 1993; Kidd *et al.*, 1993; Pieniazek *et al.*, 1990), but all other Ad have a fibre protein encoded by a single gene. In Ad2, the C-terminal 40 aa in the knob and the last shaft repeat are essential for Ad2 fibre trimerization (Hong & Engler, 1996; Novelli & Boulanger, 1991). Substitution of the Ad5 fibre by the Ad7 fibre and exchange of the Ad5 knob domain with that of Ad3 alters virus tropism, suggesting that subgroup C and subgroup B Ad fibres bind to distinct cellular receptors (Gall *et al.*, 1996; Krasnykh *et al.*, 1996; Stevenson *et al.*, 1995). The crystal structure of the Ad5 fibre knob has been determined from protein expressed in bacteria. It is a trimer with a three-bladed propeller and a surface depression (Henry *et al.*, 1994; Xia *et al.*, 1994). Each knob monomer is organized as an eight-stranded, antiparallel β-sandwich structure. Four of the β-strands (C, B, A, J) constitute the V-sheet and face towards the virion (Xia *et al.*, 1994). The

Author for correspondence: G. Santis.
Fax +44 171 403 8640. e-mail george.santis@kcl.ac.uk

0001-6058 © 1999 SGM

four other *β*-strands (G, H, I, D) form the R-sheet and face the cellular receptor (Xia *et al.*, 1994).

The common coxsackie B virus and Ad receptor (CAR) was recently identified as the major receptor for Ad5 and Ad2 fibres (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). The mouse homologue of human CAR also functions as a receptor for coxsackie B viruses and Ad (Bergelson *et al.*, 1998; Tomko *et al.*, 1997).

Control of tissue tropism and cell targeting of therapeutic genes represent a major goal of Ad vector-mediated gene transfer. The aim of this study was to identify fibre domains that determine tissue tropism without altering virus replication, assembly and infectivity. In order to generate recombinant Ad with variant fibre proteins, we developed a novel method that exploits highly efficient homologous recombination in *E. coli* (Chartier *et al.*, 1996) and permits the assessment of fibre mutants in the context of an intact Ad virion. We found that all mutants that accumulated as fibre monomers failed to assemble with a penton base and proved lethal for Ad5, which suggests that the absence of infectious virions resulted in part from a defect in fibre penton base assembly. Our results also suggest that the R-sheet of the Ad5 fibre knob monomer contains binding motifs for CAR and that *β*-strands E and F, or a region close to them, may also be involved in receptor recognition.

Methods

Construction of Ad5 fibre gene mutants and re-insertion of these mutants into the Ad5 viral genome. Deletions spanning the shaft and knob of the Ad5 fibre gene were generated first by site-specific mutagenesis using the Sculptor *in vitro* mutagenesis system (Amersham). The Ad5 *Eco*RI–*Sma*I fragment (nt 30049–33093), overlapping the entire fibre gene, was cloned in pPolyII to generate plasmid pPoly*Fibre*. *Bam*HI restriction sites (underlined) were then introduced at nucleotide positions 31689 (fibre residue 216; motif 11), 31983 (fibre residue 314; motif 17), 32244 (fibre residue 402) and 32481(fibre residue 481) using oligonucleotides 5′ catgtaacagacgacggatccctaaaacactttgacc 3′, 5′ gtttacagcttccaaacggatccaattccaaaaagcttg 3′, 5′ gataagctaactttgggattctggaccacaccagct 3′ and 5′ gaatattggaactttggattcagaaatggagatctt 3′, respectively (Fig. 1). The resulting plasmids were digested with *Nde*I (nt 31089) and *Mun*I (nt 32825) and fragments carrying the mutated fibre gene were cloned in a *Nde*I/*Mun*I-restricted pTG6590 plasmid containing the Ad5 sequences from nt 27082 to the right-end inverted terminal repeat to yield plasmids pTG8520 (*Bam*HI at nt 31689), pTG8521 (*Bam*HI at nt 31983), pTG8516 (*Bam*HI at nt 32244) and pTG8518 (*Bam*HI at nt 32481), respectively. The *Xba*I–*Bam*HI fragment of pTG8521 was replaced by the *Bam*HI–*Xba*I fragment of pTG8520 to obtain pTG8524 (deletion of nt 31690–31983; *dl*216–314). Similarly, the *Xba*I–*Bam*HI fragment of pTG8516 was replaced by the *Bam*HI–*Xba*I fragment of pTG8521 to obtain pTG8522 (deletion of nt 31983–32244; *dl*314–402). Finally, pTG8523 (deletion of nt 32244–32481; *dl*402–481) was constructed by replacing the *Xba*I–*Bam*HI fragment of pTG8518 with the *Bam*HI–*Xba*I fragment of pTG8516. An *Spe*I–*Sca*I fragment was then isolated from plasmids pTG8516, pTG8518, pTG8520, pTG8521, pTG8522, pTG8523 and pTG8524 and ligated to an *Spe*I/*Sca*I-restricted pTG6591 (Ad5 nt 21652–35935). *Bst*EII fragments (8·5 kb) of the recombinant plasmids containing the modified fibre were then isolated and recombined in *E. coli* with pTG3602 (Chartier *et al.*, 1996) to obtain plasmids pAd5F-*dl*216–314, pAd5F-*dl*314–402, pAd5F-*dl*402–481 and four additional plasmids carrying the full-length Ad5 genome with novel *Bam*HI restriction sites that resulted in glycine–serine (GS) dipeptide insertions after residues 216, 314, 402 and 481 (pAd5F^{216insGS}, pAd5F^{314insGS}, pAd5F^{402insGS} and pAd5F^{481insGS}, respectively) reconstituting these fibre modifications in the whole Ad5 genome.

In order to delete putative structural and functional domains in the Ad5 fibre knob, plasmid pPoly*Fibre* was digested with *Hin*dIII (nt 31994) and *Sma*I (nt 32991). Fragments carrying the Ad5 fibre knob were then cloned in *Hin*dIII/*Sma*I-restricted m13TG130 (Kieny *et al.*, 1983) to obtain m13F5*knob*. Strands D, G, H and I, the HI and DG loops and strands E and F were deleted as follows. Oligonucleotides 5′ tagcactccattttcgtcggatccttgaactgttcccagatat 3′, 5′ agttttgtctccgtttaaggatccacttttggagttttacc 3′, 5′ tgtttcctgtgtgtaccgttggatccttttagttttgtctccgtt 3′, 5′ gtagttgtggccagaccaggatcctgcacttgggagttgtgtc 3′, 5′ tgaaaatgacatagagtaggatcctagtgtaatggttagtgt 3′, 5′ acttgactgacaatgttggatccaaatcttataataagatg 3′ and 5′ tgtataggctgtgccttcggatccccaatattctgggtccag 3′ were annealed to m13F5*knob* single-strand template to generate m13F5*dl*D (deletion of strand D), m13F5*dl*G (deletion of strand G), m13F5*dl*H (deletion of strand H), m13F5*dl*I (deletion of strand I), m13F5*dl*HI (deletion of HI loop), m13F5*dl*DG (deletion of DG loop) and m13F5*dl*EF (deletion of strands E and F).

The *β*-strands D, G, H and I of the Ad5 fibre were substituted with the corresponding *β*-strands of Ad3 fibre by sequentially annealing oligonucleotides 5′ gtagcactccatttcgtcaaagtagagctccacgttgatactttgaactgttccagatattgg 3′ (*β*-strand D), 5′ agttttgtctccgtttaagccgaagatgtagttctcgttacttttggcagttttacc 3′ (*β*-strand G), 5′ tgtttccgtgtaccgtttagcatcacggtcacctcgagaggtttagttttgtctccgtttaag 3′ (*β*-strand H) and 5′ gtagttgtggccagaccagctccacagaaaggtcattacgtatgcacttggagttgtgtctc 3′ (*β*-strand I) to m13F5*knob* single-strand template to generate m13V5R3 (substitution of Ad5 with Ad3 R-sheet).

In order to reconstitute these fibre gene mutations in the Ad5 genome, a modification of our previously described method (Chartier *et al.*, 1996) was developed. A *Hin*dIII–*Sma*I fragment was isolated from these phage and was recombined in *E. coli* with pTG4609 linearized by *Bst*BI to obtain plasmids pAd5F-*dl*D, pAd5F-*dl*EF, pAd5F-*dl*G, pAd5F-*dl*H, pAd5F-*dl*I, pAd5F-*dl*DG and pAd5F-*dl*HI and pAd5F-*R3*. Plasmid pTG4609 contains the full Ad5 genome in which a unique *Bst*BI restriction site was introduced at nt 32940, downstream of the fibre stop codon.

Phenotypic analysis of fibre protein mutants. Isolation of full-length Ad2, full-length Ad5, full-length Ad3 genes and the Ad3 knob was described in a previous study (Hong *et al.*, 1997). Recombinant full-length fibre and fibre knob of adenovirus serotypes Ad2 (F2-WT and F2-knob, respectively) and Ad3 (F3-WT and F3-knob, respectively), wild-type Ad5 fibre (F5-WT), mutant Ad5 fibre proteins F5-*dl*216–314, F5-*dl*314–402, F5-*dl*402–481, F5-*dl*G, F5-*dl*D, F5-*dl*EF, F5-*dl*I, F5-*dl*DG, F5-*dl*HI and V5R3 (Table 1) and full-length Ad5 penton base protein (Pb2-WT) were expressed in *Spodoptera frugiperda* cells (Sf9), using recombinant *Autographa californica* nucleopolyhedroviruses (AcMNPV). The baculovirus intermediate transfer vector and cloning strategies have been described previously in detail (Novelli & Boulanger, 1991). The AcMNPV clone expressing the Ad2 knob domain was obtained from J. Chroboczek (CNRS/CEA, Grenoble, France).

Wild-type (WT) and mutant Ad5 fibre proteins were analysed phenotypically with respect to (i) trimerization, (ii) glycosylation and (iii) assembly in Sf9 cells with recombinant penton base to form a penton. Oligomerization was assessed by means of non-denaturing SDS–PAGE (NDS–PAGE) and denaturing SDS–PAGE. NDS–PAGE differed from

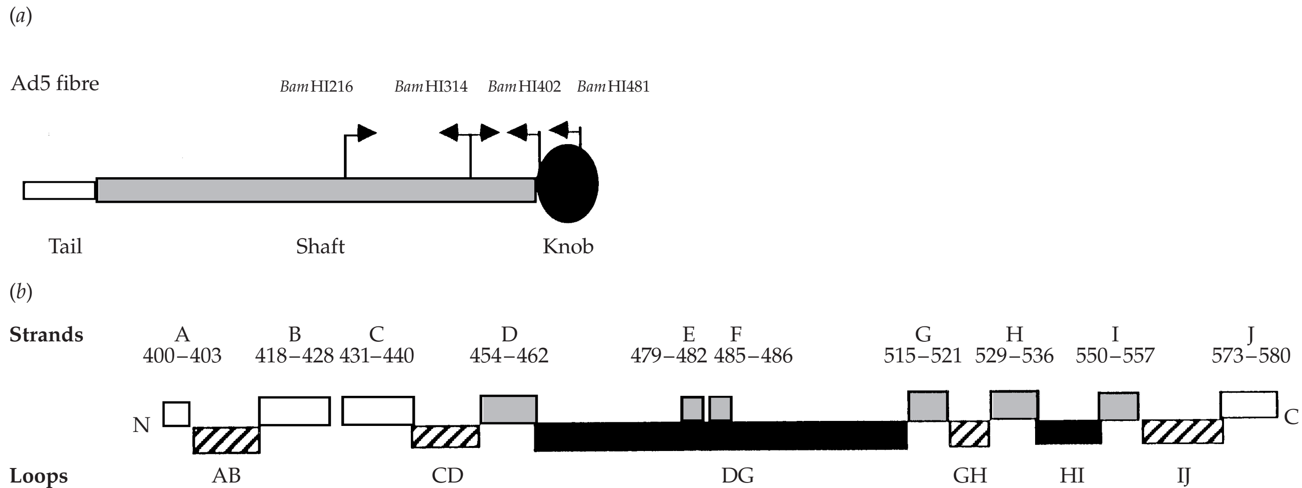

Fig. 1. (*a*) Schematic representation of Ad5 fibre. The Ad5 fibre consists of three domains, the N-terminal tail (aa 1–46), the midsection shaft domain (aa 47–399) and the C-terminal knob domain (aa 400–581). Numbers correspond to novel *Bam*HI restriction sites that were introduced in the fibre shaft and knob domains. (*b*) Schematic representation of functional domains in Ad5 fibre knob. Boxes indicate putative functional domains (Xia *et al*., 1994) as follows: grey boxes, *β*-strands in the V-sheet (D, E, F) and in the R-sheet (G, H, I) that were deleted; black boxes, loops that were deleted as part of site-directed mutagenesis; hatched boxes, loops; and white boxes, strands that were not mutagenized in these experiments. Numbers correspond to Ad5 fibre residues.

Table 1. Phenotypic analysis of mutant Ad5 fibres

WT and mutant Ad5 fibre proteins were analysed phenotypically with respect to (i) trimerization, (ii) assembly in Sf9 cells with recombinant penton base to form the penton and (iii) glycosylation. Transfection of mutant Ad5 genomes into 293 cells was by the calcium phosphate method according to the manufacturer's instructions. Recombinant viruses were plaque-purified twice (Graham & Prevec, 1992).

Ad5 fibre mutant	Quaternary structure	Assembly with penton base	Glycosylation	Virus titre (p.f.u. per cell)
WT	Trimer	Yes	Glycosylated	$3{\cdot}7\times10^{10}$
F5-*dl*216–314	Trimer	Yes	Glycosylated	$2{\cdot}6\times10^{8}$
F5-*dl*314–402	Monomer	No	Non-glycosylated	No plaques
F5-*dl*402–481	Monomer	No	Non-glycosylated	No plaques
F5-*dl*D	Monomer	No	Non-glycosylated	No plaques
F5-*dl*G	Monomer	No	Non-glycosylated	No plaques
F5-*dl*I	Monomer	No	Non-glycosylated	No plaques
F5-*dl*EF	Trimer	Yes	Glycosylated	$2{\cdot}3\times10^{9}$
F5-*dl*DG	Monomer	No	Non-glycosylated	No plaques
F5-*dl*HI	Monomer	No	Non-glycosylated	No plaques
V5R3	Monomer	No	Non-glycosylated	No plaques
F5-*dl*H	Unstable protein	–	–	No plaques
$F5^{216insGS}$	Not analysed	–	–	$1{\cdot}7\times10^{9}$
$F5^{314insGS}$	Not analysed	–	–	$1{\cdot}5\times10^{9}$
$F5^{402insGS}$	Not analysed	–	–	$7{\cdot}2\times10^{9}$
$F5^{481insGS}$	Not analysed	–	–	$3{\cdot}5\times10^{9}$

SDS–PAGE in that the samples were not denatured by boiling in SDS sample buffer prior to electrophoresis. The immunoblotting reaction of fibre was performed using anti-fibre rabbit polyclonal antibody and a phosphatase-labelled or ^{125}I-labelled (Amersham) anti-rabbit IgG conjugate as previously described (Novelli & Boulanger, 1991). Glycosylation of recombinant fibres was assessed by immunoreaction on blots with MAb RL2, which is specific for *O*-linked GlcNAc residues (Mullis *et al*., 1990). Assembly of fibre with recombinant penton base *in vivo* was carried out by co-expressing two recombinant AcMNPV in the same Sf9 cells. The presence of the penton capsomer was investigated in cell lysates harvested at 40 h post-infection, with the samples processed and analysed by PAGE in native conditions (Karayan *et al*., 1994).

Expression and purification of recombinant WT and mutated Ad fibres. Expression and purification of WT and mutated Ad fibres in Sf9 cells were performed as previously described (Novelli & Boulanger, 1991). The concentration of WT and mutant fibres was estimated by SDS–PAGE analysis of fibre samples run in gels with a range of BSA samples of known protein content, and OD comparison was performed by gel scanning using an automatic densitometer system (REP–EDC; Helena Laboratories). The concentration of all fibre mutants was normalized to the WT Ad5 fibre concentration as previously described (Karayan *et al.*, 1997). To express the WT Ad5 fibre knob in bacteria, Ad5 sequences from nt 32 197–32 783, corresponding to the Ad5 fibre knob and 15 residues of the terminal repeating unit of the shaft, were synthesized by PCR on pTG3602 plasmid DNA using primers 5′ cccgaattctatgggtgccattacagtaggaaa 3′ (5′ oligonucleotide) and 5′ cccaagctattcttgggcaatgtatga 3′ (3′ oligonucleotide), which contain a *Hin*dIII and an *Eco*RI site, respectively. The amplified product was ligated in a *Hin*dIII/*Eco*RI-restricted pKK233-3, an IPTG-inducible expression vector (Pharmacia) to yield pKKF5*knob*. Overnight culture (1 ml) was used to seed 50 ml fresh media; 50 ml cultures were grown overnight and then used to seed 500 ml fresh media, which were then grown for 3 h before induction with IPTG at 1 mM final concentration. Cells were harvested at 6 h and washed in 20 mM Tris (pH 7·8), 1 mM EDTA, then pelleted. Extraction and purification of recombinant fibre knob was carried out as previously described (Henry *et al.*, 1994).

Cell binding competition assays. The capacity for cell attachment of recombinant mutant fibres to functional receptors on CHO–CAR cells (CHO cells that have been transfected with CAR cDNA and express functional CAR) was estimated from cell binding competition assays between Ad5Luc3 and recombinant fibres using the level of luciferase gene expression as the end-point assay. This assay measures fibre attachment to functional cell surface receptors and dissociates virus attachment from internalization because at low temperatures only virus–cell attachment occurs, whereas endocytosis requires physiological temperatures. Ad5Luc3, obtained from F. Graham (McMaster University, Hamilton, Canada), is a replication-competent virus, which contains the luciferase gene under the control of the SV40 early promoter inserted into the E3 region of the Ad5 genome. CHO–CAR cells were infected at constant m.o.i. of Ad5Luc3 (4 p.f.u. per cell) in the presence of a large excess of recombinant proteins. Ad5Luc3 was pre-incubated with recombinant proteins at room temperature and the mixture was added to CHO–CAR cells pre-cooled on ice. After incubation for 1 h at 0 °C, unabsorbed virus was rinsed off, the cell monolayers were covered with pre-warmed medium, transferred to 37 °C and further incubated at that temperature for 18 h, then processed for the luciferase assay. Luciferase activity, expressed in relative light units (RLU), was assayed in cell lysates using luciferase substrate solution.

The efficiency and affinity of recombinant full-length fibre proteins of Ad2, Ad5 and Ad3, of their corresponding fibre knobs, and of Ad5 mutant fibres for CHO–CAR cell receptors was estimated on lysates of cells (10^5 cells per sample) infected with Ad5Luc3 in the presence of increasing amounts of recombinant proteins. The efficiency with which mutant Ad5 fibre bound to CHO–CAR cell receptors was assessed by measuring the reduction in luciferase activity in the presence of mutant fibre protein concentrations that were at least 100-fold higher than the WT Ad5 fibre protein concentration required to almost completely abolish Ad5Luc3 infection of CHO–CAR cells. The relative affinities of WT and mutant fibre binding to CAR were assessed by measuring the amount of each protein required to achieve 50% maximal inhibition of luciferase activity (IC_{50}). The IC_{50} for each mutant (in ng) was calculated as the protein concentration required to obtain 50% maximal inhibition.

Antibodies. Rabbit antisera against Ad2 virion proteins and against electrophoretically purified recombinant Ad2 fibre protein were all laboratory-made (Hong *et al.*, 1997; Novelli & Boulanger, 1991). The occurrence of carbohydrate residues was analysed by immunoreaction on blots with MAb RL2, specific for *O*-linked GlcNAc residues (Mullis *et al.*, 1990). MAb RmcB, which recognizes CAR protein, was described in a previous study (Bergelson *et al.*, 1997). MAb 4D2.5, which recognizes the highly conserved fibre tail motif FNPVYP (Hong & Boulanger, 1995), was obtained from J. Engler (UAB at Birmingham, AL, USA).

Mammalian cells and DNA transfections. 293 embryonic kidney cells and HeLa cells were obtained from ATCC. They were maintained in Dulbecco's medium supplemented with 10% foetal calf serum. CHO and CHO–CAR cells (Bergelson *et al.*, 1997) were maintained in Dulbecco's medium supplemented with 10% foetal calf serum. 293 cells were transfected by the calcium phosphate method according to the manufacturer's instructions. Recombinant viruses were plaque-purified twice and the mutations were confirmed by restriction enzyme analysis of viral DNA (Graham & Prevec, 1992).

Results

Mutagenesis of the Ad5 fibre gene

The crystal structure of Ad5 fibre knob revealed serotype-specific regions that might bind to distinct receptors (Xia *et al.*, 1994). We sought to investigate these regions by site-specific mutagenesis of Ad5 fibre protein (Table 1). We introduced 14 mutations, including deletions in the putative receptor binding region of the protein (Xia *et al.*, 1994) (deletion of the DG and HI loops and of β-strands D, E, F, G, H and I), the deletion of 78 aa from residues 402–481 in the knob domain, and two large deletions in the shaft spanning aa 216–314 and 314–402 (Fig. 1). In addition, β-strands G, H, I and D in the R-sheet of Ad5 fibre were substituted with the corresponding β-strands of Ad3 to generate the chimaeric Ad5 fibre V5R3. The nucleotide sequences of all mutant fibres were determined; each contained the appropriate mutation without any additional sequence alterations.

Phenotypic analysis of recombinant mutated fibres

In order to address the effect of these mutations on capsomere assembly, the ten deletion mutants and the Ad5/Ad3 chimaera were assessed phenotypically as recombinant proteins in baculovirus-infected cells.

Oligomerization of Ad fibres expressed in insect cells was assessed by their electrophoretic mobility in non-denaturing gels (NDS–PAGE) and by their ability to bind with recombinant penton base to form stable penton structures. In NDS–PAGE analyses, fibre trimers are visible as multiple discrete bands in the 170–180 kDa zone, representing intermediate steps in assembly or partially unfolded trimers. Free unassembled monomers are also found, but they account for less than 10% of the total protein.

We found that all Ad5 fibre mutants, apart from the mutant lacking strand H (fibre F5-*dl*H), were expressed at high levels in

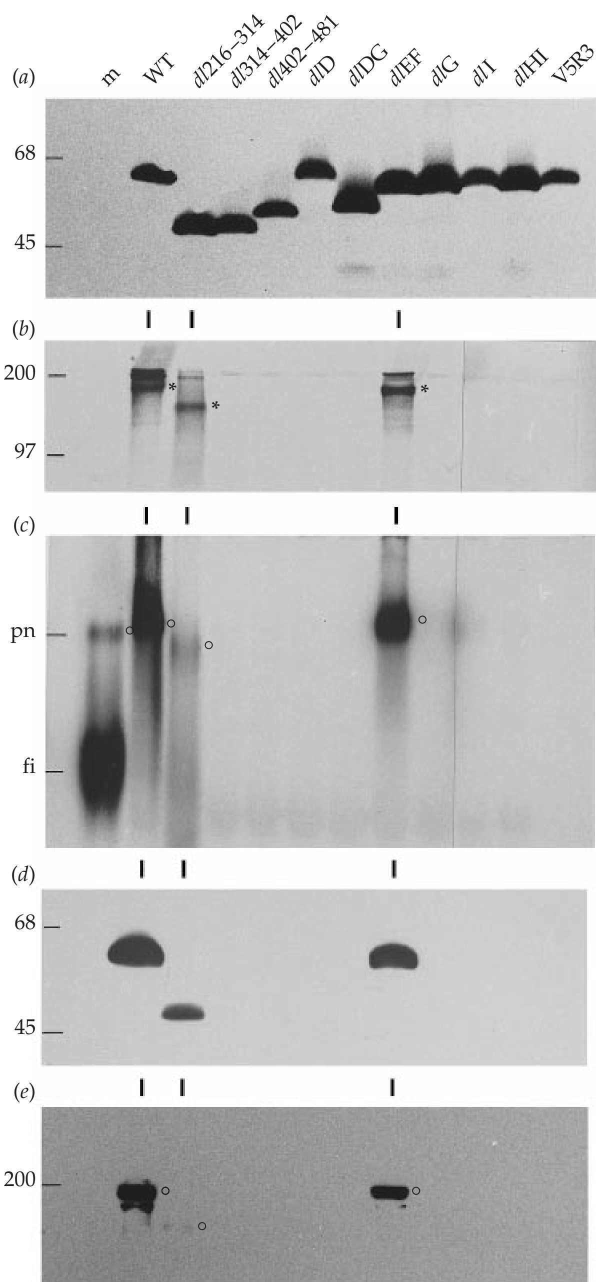

Fig. 2. (*a*) Expression of recombinant fibre in baculovirus-infected Sf9 cells. SDS–PAGE analysis of whole-cell lysates showing the level of expression of WT and mutant fibre proteins. (*b*) Oligomerization of recombinant fibre proteins. Immunoblot of WT and mutant fibre cell lysates electrophoresed under NDS–PAGE conditions. The cell lysates were not denatured by boiling to preserve the oligomeric structure of the fibre proteins. The blots were reacted with anti-fibre rabbit antibody and phosphatase-conjugated secondary antibody. Trimeric fibres (180 kDa) are indicated by asterisks. Monomeric fibres were visualized but are not shown. On the left, the positions of the molecular mass markers for myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa) are shown. The blots in (*a*) and (*b*) were reacted with MAb 4D2.5 and peroxidase-conjugated secondary antibody. (*c*) Assembly of penton capsomer *in vivo* in Sf9 cells co-expressing WT penton base with WT and mutant fibres. Non-denatured cell lysates were analysed by PAGE under native conditions and immunoblotted. The assembled penton capsomers (pn), indicated by open circles, appear as discrete bands and free fibres (fi) appear as diffuse bands. Ad2 capsid protein markers, fibre and penton are shown in the left lane. The absence of a detectable fibre band in the non-trimeric mutants was due to their lack of solubility as previously shown (Karayan *et al*., 1994). Autoradiography of the blot reacted with anti-fibre rabbit antibody and ^{125}I-labelled secondary antibody. (*d*) and (*e*) Glycosylation status of recombinant fibre proteins in baculovirus-infected Sf9 cells. Cell lysates were analysed by (*d*) SDS–PAGE and (*e*) NDS–PAGE, followed by immunoblotting and reaction with anti-*O*-GlcNAc–peptide MAb (RL2). WT fibre monomers migrate with an apparent molecular mass of 62 kDa in SDS gel, fibre trimers as 180 kDa. *O*-GlcNAc fibre trimers are indicated by open circles. m, Marker lane.

Sf9 cells (Fig. 2*a*; Table 1). Ad5 fibre F5-*dl*H was not detected (data not shown), probably because of proteolysis of an incorrectly folded mutant fibre protein at a preferred cleavage site. Ad5 fibre F5-*dl*EF, which lacks strands E and F (residues 479–486), formed stable trimers and assembled with the penton base to form stable penton protein (Fig. 2*b*, *c*). These findings demonstrate that strands E and F in the V-sheet are dispensable for fibre assembly and that the hydrogen bonds between residues 482 (E strand) and 485 (F strand), noted in the crystal structure (Xia *et al*., 1994), are not critical for trimerization. Fibre F5-*dl*216–314, which has a deletion of aa 216–314 within the shaft, also formed stable trimers and penton structures, but its ability to assemble with penton base appeared significantly impaired, suggesting that this region in the shaft of the Ad5 fibre influences the efficiency or stability of the interaction between the penton base and the tail of the fibre protein (Fig. 2*b*, *c*). The chimaeric fibre V5R3 and fibres lacking aa 314–402 (90 aa deletion spanning the shaft and shaft–knob junction), aa 403–481 (78 aa deletion spanning the knob domain), strands D, G, I and the HI and DG loops accumulated exclusively or predominantly ($>90\%$) as monomers and failed to assemble with the penton base, indicating that these domains are all critical for fibre oligomerization (Fig. 2*b*, *c*).

The deletion of the terminal 90 aa in the shaft includes deletion of the last terminal shaft repeat that was recently shown to be essential for trimerization of Ad2 fibre (Hong & Engler, 1996) and of the highly conserved strand A. Mutant fibre F5-*dl*402–481, which also accumulated as a monomer, lacks strands B, C and D and part of β-strand E. These strands constitute most of the core of the fibre knob and are therefore essential for the correct folding and assembly of the fibre trimer. Strands D, G and I and the DG and HI loops are all within the R-sheet. Of these, only strand G is thought to be involved in trimerization (Xia *et al*., 1994). Our results confirm the critical role of strand G in trimerization, but also demonstrate that strands D and I and the DG and HI loops are also critical. Deletion of β-strands D, G and I may result in incorrect folding of the knob monomers, whereas deletion of one of the surface loops may impede the correct orientation of the β-sandwich motif.

In vivo, Ad fibre protein is glycosylated. The functional significance of this post-translational modification is unknown.

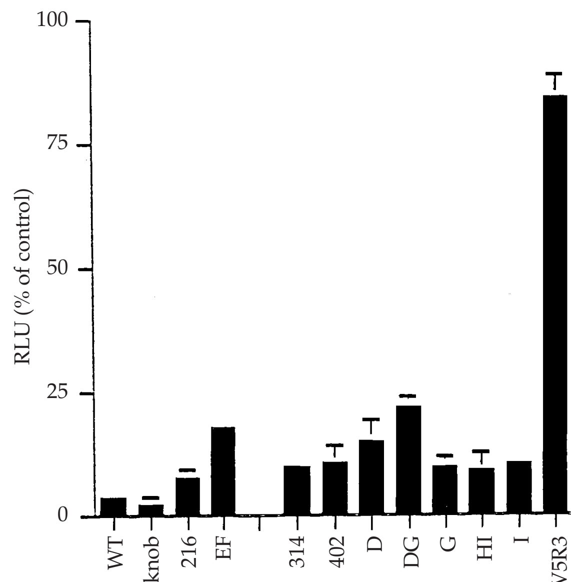

Fig. 3. Reduction in luciferase activity in CHO–CAR in the presence of maximal amounts of WT and mutant Ad5 fibres (F5-WT, 1416 ng; F5-knob, 1500 ng; F5-*dl*216–314, 637 ng; F5-*dl*EF, 1650 ng; F5-*dl*314–402, 1095 ng; F5-*dl*402–481, 906 ng; F5-*dl*D, 1057·5 ng; F5-*dl*DG, 831 ng; F5-*dl*G, 1341 ng; F5-*dl*I, 1110 ng; F5-*dl*HI, 1080 ng; and V5R3, 1240·5 ng). Cells were infected at constant m.o.i. of Ad5Luc3 in the presence of a large excess of recombinant full-length Ad5 fibre proteins. Ad5Luc3 was pre-incubated with recombinant proteins at room temperature and the mixture added to CHO–CAR cells pre-cooled on ice. After incubation for 1 h at 0 °C, unabsorbed virus was rinsed off, the cell monolayers were covered with pre-warmed medium, transferred to 37 °C and further incubated at that temperature for 18 h, then processed for the luciferase assay. Luciferase activity, expressed in relative light units (RLU) was assayed in cell lysates using luciferase substrate solution. Results are expressed as percentages of the control cells (i.e. no recombinant fibre, 100%). The data presented are means and standard errors of the means ($n = 3$) of three representative experiments. WT, knob, 216 and EF correspond to full-length WT Ad5 fibre, fibre knob and the trimeric mutant fibres F5-*dl*216–314 and F5-*dl*EF respectively; 314, 402, D, DG, G, HI, I and V5R3 correspond to the monomeric fibre mutants F5-*dl*314–402, F5-*dl*402–481, F5-*dl*D, F5-*dl*DG, F5-*dl*G, F5-*dl*HI, F5-*dl*I and the chimaeric monomeric Ad5 fibre V5R3, respectively.

Immunoblotting with MAb RL2 gave positive results with recombinant fibre F5-*dl*EF, indicating that this mutant fibre was glycosylated (Fig. 2). In contrast, RL2 failed to recognize any of the monomeric fibre mutants and recognized the trimeric protein F5-*dl*216–314 with reduced efficiency (approximately 10-fold; Fig. 3). These findings suggest that glycosylation of Ad5 fibre occurs after stable trimers are formed. It is unlikely that glycosylation is required for trimerization since recombinant fibre knob expressed in bacteria is also a trimer (Xia *et al.*, 1994; this study).

Generation of viral genomes with mutations in the fibre gene

We generated recombinant plasmids containing the full-length Ad5 genome with all the fibre gene modifications described above and four additional plasmids carrying the full-length Ad5 genome with novel *Bam*HI restriction sites that resulted in a glycine–serine (GS) dipeptide insertion after residues 216 (pAd5F^{216insGS}), 314 (pAd5F^{314insGS}), 402 (pAd5F^{402insGS}) and 481 (pAd5F^{481insGS}), respectively. In order to facilitate this process, we developed a rapid two-step cloning strategy that exploits highly efficient homologous recombination in *E. coli* (Chartier *et al.*, 1996) and permits the assessment of fibre mutants in the context of an intact Ad virion. Transfection of these Ad5 viral genomes into 293 cells allowed the recovery of infectious virus particles for the four GS insertion mutants as well as for two deletion mutants pAd5F-*dl*216–314 and pAd5F-*dl*EF (Table 1). The virus titre of pAd5F-*dl*216–314 was $2{\cdot}6 \times 10^8$ p.f.u. per cell compared to the WT titre of $3{\cdot}7 \times 10^{10}$ p.f.u. per cell (Table 1). This is compatible with our observation that the ability of fibre F5-*dl*216–314 to assemble with the penton base was significantly impaired. In contrast, all other fibre mutants that accumulated as fibre monomers also prevented the production of infectious viruses (Table 1). These findings indicate that aa 216–314 within the shaft and β-strands E and F in the knob do not interfere with virus assembly and infectivity, whereas the region spanning the shaft and the shaft–knob junction, most of the V-region of the knob and strands D, G, H and I, as well as the DG and HI loops, are required for the viability of Ad5 virions.

Cell binding capacity of Ad5 fibre mutants for functional receptors present on CHO–CAR cells

Failure to obtain viable adenovirions from fibre-modified viral DNA could be due to an adverse effect of these fibre mutations on cell receptor recognition. This was evaluated in cell binding competition experiments with adenovirions encoding luciferase (Ad5Luc3) using CHO–CAR cells by measuring the reduction in luciferase activity in the presence of increasing amounts of WT and mutant Ad5 fibres. This assay measures fibre attachment to functional cell surface receptors and dissociates virus attachment from internalization because, at low temperatures, only virus–cell attachment occurs, whereas endocytosis requires physiological temperatures. Ad5Luc3-mediated luciferase expression also depends on endosome penetration and transport to the cell nucleus. These steps in the virus life-cycle were kept constant by using Ad5Luc3 at constant m.o.i. in all experiments.

The efficiency with which mutant Ad5 fibre bound to CHO–CAR cell receptors was assessed by measuring the reduction in luciferase activity in the presence of maximal concentrations (100-fold higher concentration than the WT Ad5 fibre protein concentration required to almost completely abolish Ad5Luc3 infection) (Fig. 3). The relative affinities of WT and mutant fibre binding to CAR were assessed by measuring the amount of each protein required to achieve 50% of maximal inhibition of luciferase activity (IC_{50}) (Table 2).

CHO–CAR cells were infected with Ad5Luc3 at a constant m.o.i. (4 p.f.u. per cell). In the absence of competing Ad fibres, luciferase gene expression in CHO–CAR cells infected with

Table 2. Affinity of WT and mutant Ad fibres for CHO–CAR cell receptors

The affinity of recombinant WT and mutant fibres for CHO–CAR cell receptors was estimated by using the luciferase assay on lysates of cells (10^5 cells per sample) infected with Ad5Luc3 in the presence of increasing amounts of recombinant proteins. The IC_{50} for each mutant (in ng) was calculated as the protein concentration required to obtain 50% maximal inhibition. ND, Not done.

Ad fibre	IC_{50} (ng per 10^5 cells) for CHO–CAR cell receptors Mean (SD)	Ratio to WT
WT fibres		
F5-WT	33·5 (2·8)	1·0
F5-knob	42·7 (3·1)	1·3
F2-WT	50·0 (5·6)	1·5
F2-knob	ND	–
F3-WT	829·5 (37·6)	24·8
F3-knob	1250·0 (60·6)	37·3
Trimeric mutant fibres		
F5-*dl*216–314	31·4 (3·3)	0·9
F5-*dl*EF	364 (40·8)	10·9
Monomeric mutant fibres		
F5-*dl*314–402	76·3 (5·4)	2·2
F5-*dl*402–481	180 (14·7)	5·3
F5-*dl*D	171·6 (29·0)	5·1
F5-*dl*G	288 (47·0)	8·6
F5-*dl*I	188 (78·0)	5·6
F5-*dl*DG	180·6 (23·0)	5·4
F5-*dl*HI	227·5 (20·0)	6·8
V5R3	827·0 (57·6)	24·7

4 p.f.u. per cell of Ad5Luc3 was 38923 RLU compared to 428 RLU obtained with CHO cells infected with Ad5Luc3 at the same m.o.i.

At the highest concentration of WT Ad5 fibre, the luciferase activity was inhibited by 98% in CHO–CAR cells (Fig. 3). Fibre F5-*dl*216–304 (trimeric in nature) gave comparable reductions in luciferase activity (Fig. 3). Also, the IC_{50} of F5-*dl*216–304 fibre was similar to that of the WT Ad5 fibre (Table 2), which suggests that deletion of aa residues 216–314 in the shaft does not alter the efficiency or affinity with which Ad5 fibre binds to CAR. Although fibre F5-*dl*EF competed efficiently with Ad5Luc3 for CHO–CAR cell surface ligands (Fig. 3), its IC_{50} for CAR was 10-fold higher than that of the WT Ad5 fibres for the same receptor (Table 2). The quaternary structure of F5-*dl*EF, assessed by its capacity to trimerize, assemble with recombinant penton base protein to form intact pentons and to be glycosylated, was the same as that of WT fibre, which indicates that β-strands E and F, or a region close to them, may be involved in receptor recognition.

Fibre F5-*dl*314–402 (monomeric knob) reduced luciferase expression in CHO–CAR cells by more than 90% (Fig. 3), which indicates that trimerization is not essential for receptor recognition. This is supported by the finding that all other monomeric fibres, including those deleted of individual loops and strands in the knob, competed efficiently with Ad5Luc3 for CAR (Fig. 3). The IC_{50} of F5-*dl*314–402 was 2-fold higher than the IC_{50} of WT fibre, which suggests that F5-*dl*314–402 binds to CAR with reduced affinity. This may be explained by differences in avidity for the receptor between monomeric (F5-*dl*314–402) and trimeric (WT) molecules. The IC_{50} values of monomeric fibres deleted of individual strands and loops in the knob domain for CAR were higher than those of WT Ad5 fibre and fibre F5-*dl*314–402 (Table 2). This is probably due to altered conformation of the fibre knob. Alternatively, the deletion of specific strands or loops in the R-sheet reduced the affinity of mutant fibres for CAR by altering receptor binding sites. The role of β-strand H in receptor binding could not be assessed because recombinant fibres deleted of this strand were not detected in insect cells.

Chimaeric fibre V5R3, in which β-strands D, G, H and I of Ad5 fibre were replaced by the corresponding β-strands of Ad3 fibre and which accumulated as a monomer, failed to compete with Ad5Luc3 for CHO–CAR cell receptors (Fig. 3). This is in contrast to monomeric fibres deleted of individual D, G and I strands that retained the capacity to block binding to CAR when used at high concentrations. In addition, there was no significant inhibition of luciferase activity in the presence of maximal amounts of Ad3 fibre and Ad3 fibre knob, respectively (data not shown). These findings suggest that Ad3 fibre and mutant V5R3 do not bind efficiently to CAR and that the R-sheet of the Ad5 fibre knob contains binding motifs for CAR. Ad3 infection of HeLa cells was almost totally abolished by high concentrations of Ad3 fibre, Ad3 fibre knob and by V5R3 fibre (data not shown). This demonstrates that monomeric V5R3 fibre was functional for binding to Ad3 cell receptor(s) and that the exchange of sheets between Ad3 and Ad5 did not significantly alter the conformation of the V5R3 monomer.

Discussion

In this study, we assessed the molecular determinants of receptor binding and structural characterization of Ad5 fibre by deletion analysis of putative structural and functional domains of the protein.

We found that β-strands D, G and I and the DG and HI loops in the R-sheet of the Ad5 fibre monomer are all essential for stable trimer formation. Of these, only strand G was thought to be involved in trimerization (Xia *et al*., 1994). The deletion of entire strands and loops in the R-sheet of the fibre knob may affect protein structure globally or, alternatively, remove specific regions that are critical for trimerization. The removal of β-strands D, G and I, which participate in hydrogen bonding interactions in the β-sandwich motif (Xia *et al*., 1994), may result in incorrect folding of the knob monomers which then fail to form stable trimers. The deletion of a surface loop

that does not participate in hydrogen bonding interactions may disrupt the structure of the monomer by impeding the correct orientation of the *β*-sandwich motif. In addition, deletion of the DG loop may render the fibre knob non-trimeric by deletion of the highly conserved hydrophobic region between residues 494 and 505 that contributes to the hydrophobic core of the monomer.

Previous studies have shown that Ad2 and Ad5 fibres contain multiple monosaccharide *O*-GlcNAc residues and that mutant baculovirus- and vaccinia virus-expressed Ad2 fibres contain *O*-GlcNAc residues even when they accumulated only as monomers (Hong & Engler, 1996; Novelli & Boulanger, 1991). This would indicate that, in Ad2 fibre, glycosylation occurs despite incorrect folding of the protein. In contrast, we found that all monomeric baculovirus-expressed Ad5 fibres were not glycosylated. This difference between Ad2 and Ad5 fibres may be accounted for by the fact that Ad2 fibre contains more glycosylation sites than Ad5.

Attachment of WT and mutant recombinant Ad fibres to CAR was assayed in CHO–CAR cells by competition with Ad5Luc3. These experiments, which were performed at 0 °C, a temperature that allows attachment but not internalization of the virus, assessed binding of Ad fibres to functional cell surface receptors.

We found that fibres of subgroup C (Ad5, Ad2) but not of subgroup B1 (Ad3) competed efficiently for CAR binding. It was previously shown that Ad35 (subgroup B2) fibres also failed to bind to CAR (Bergelson *et al*., 1997). These findings are compatible with previous studies which showed that Ad5 and Ad3 fibres bind to distinct cellular receptors. They are also in agreement with those of Roelvink *et al*. (1998) who recently found that CAR is a receptor for subgroup A, C, D, E and F Ad fibres but not for fibres of subgroup B Ad like serotype 3 (Roelvink *et al*., 1998). Because of the ability of multiple Ad as well as coxsackie B viruses to bind to CAR, it has been suggested that the Ad fibre–receptor interaction is not the sole determinant of virus tissue tropism. It has been hypothesized that the length of the Ad fibre shaft is a major determinant of Ad attachment strategy; eight or less *β*-repeats in the shaft result in attachment being enhanced by an interaction between the penton base protein and cell surface integrins (Roelvink *et al*., 1998). The effect of reducing the length of the shaft domain of the Ad5 fibre on the interaction between adenovirions and cell surface virus receptors is unknown. We found that a trimeric Ad5 mutant fibre that contains 16 *β*-repeats in the shaft instead of the 22 *β*-repeats present in WT Ad5 fibre competed efficiently with Ad5 for CAR. This would imply that the deletion of six *β*-repeats did not alter significantly binding to CAR. We also found that this shortened fibre had no effect on virus stability and infectivity which would imply that this shaft deletion had no major effect on virus interaction with surface receptors on 293 cells.

Two possible receptor binding modes have been proposed for Ad5 fibre knob (Xia *et al*., 1994). In the first, receptor binding is through an interaction between the cellular receptor and the central surface depression of the Ad5 fibre trimer. Alternatively, binding to the cellular receptor employs the surface formed by the R-sheet and the HI loop on each blade of the trimers. In this case, three receptor-binding sites could be utilized by each fibre trimer. We found that Ad5 binding to CAR is not critically dependent on the trimeric structure of the protein since Ad5 fibre monomers were shown to compete efficiently for functional receptors on CHO–CAR cells. This would suggest that the molecular determinants of binding to CAR are present in Ad5 monomers as well as trimers and that they may be arranged similarly in Ad5 fibre monomers and trimers. Although mutant Ad5 fibre monomers bound to CHO–CAR cells efficiently, their affinity for CAR was reduced compared to WT Ad5 fibre. This may be explained by differences in avidity for the receptor between monomeric (F5-*dl*314–402) and trimeric WT knob domains (WT Ad5 fibre) or, for mutant fibres deleted of individual strands and loops in the R-sheet, due to altered conformation of the fibre knob.

Our cell binding competition experiments also revealed regions in the Ad5 fibre knob that may be involved in receptor binding. Firstly, substitution of the R-sheet of Ad5 fibre with the corresponding region in Ad3 abolished binding to CAR, even at the highest competing protein concentration used. This would suggest that the R-sheet of Ad5 fibre appears to contain binding motifs for CAR and may represent a major determinant of receptor recognition and usage by the virus. Secondly, mutant fibre F5-*dl*EF bound efficiently but with reduced affinity to CAR. Since its quaternary structure was indistinguishable from the quaternary structure of WT Ad5 fibre, it is possible that the region defined by *β*-strands E and F, or a region close to it, may also be involved in receptor recognition.

G. Santis was a recipient of an Advanced Fellowship for Clinical Graduates by the Wellcome Trust and Saw See Hong a recipient of a post-doctoral Fellowship by the Association Française de Lutte contre la Mucoviscidose (AFLM). This work was supported by grants from the Wellcome Trust and the Special Trustees for Guy's and St Thomas' Hospital (G. Santis), Convention Industrielle pour la Formation par la Recherche (V. Legrand; CIFRE grant 512/94) and the AFLM and the Association Française contre les Myopathies (AFM) (P. Boulanger; AFLM-970004). J. Bergelson is supported by grants from the National Institutes of Health and an Established Investigators award from the American Heart Association. We are grateful to Bernard Gay for the photographic illustrations.

References

Bai, M., Campisi, L. & Freimuth, P. (1994). Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. *Journal of Virology* **68**, 5925–5932.

Belin, M. T. & Boulanger, P. (1994). Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface. *Journal of General Virology* **74**, 1485–1497.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg,

R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323.

Bergelson, J. M., Krithivas, A., Celi, L., Droguett, G., Horwitz, M. S., Wickham, T., Crowell, R. L. & Finberg, R. W. (1998). The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *Journal of Virology* **72**, 415–419.

Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A. & Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *Journal of Virology* **70**, 4805–4810.

Davison, A. J., Telford, E. A. R., Watson, M. S., McBride, K. & Mautner, V. (1993). The DNA sequence of adenovirus type 40. *Journal of Virology* **234**, 1308–1316.

Davison, E., Diaz, R. M., Hart, I. R., Santis, G. & Marshall, J. F. (1997). Integrin $\alpha 5\beta 1$-mediated adenovirus infection is enhanced by the integrin activating antibody TS2/16. *Journal of Virology* **71**, 6204–6207.

Defer, C., Belin, M.-T., Caillet-Boudin, M.-L. & Boulanger, P. (1990). Human adenovirus–host cell interactions, comparative study with members of subgroups B and C. *Journal of Virology* **64**, 3661–3673.

Gall, J., Kass-Eisler, A., Leinwand, L. & Falck-Pedersen, E. (1996). Adenovirus type 5 and 7 capsid chimera, fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *Journal of Virology* **70**, 2116–2123.

Graham, F. L. & Prevec, L. (1992). Adenovirus based expression vectors and recombinant vaccines. In *Vaccine, New Approaches to Immunological Problems*, pp. 363–390. Edited by R. W. Ellis. UK; Butterworth-Heinemann.

Green, N. M., Wringley, N. G., Russell, W. C., Martin, S. R. & MacLachlan, A. D. (1983). Evidence of a repeating cross-β sheet structure in the adenovirus fibre. *EMBO Journal* **2**, 1357–1365.

Hennache, B. & Boulanger, P. (1977). Biochemical study of KB-cell receptor for adenovirus. *Biochemistry Journal* **166**, 237–247.

Henry, L. J., Xia, D., Wilke, M. E., Deisenhofer, J. & Gerard, R. D. (1994). Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. *Journal of Virology* **68**, 5239–5246.

Hong, S. S. & Boulanger, P. (1995). Protein ligands of the human adenovirus type 2 outer capsid identified by biopanning of a phage-displayed peptide library on separate domains of wild-type and mutant penton capsomeres. *EMBO Journal* **14**, 4714–4727.

Hong, J. & Engler, J. (1996). Domains required for assembly of adenovirus type 2 fiber trimers. *Journal of Virology* **70**, 7071–7078.

Hong, S. S., Karayan, L., Tournier, J., Curiel, D. T. & Boulanger, P. A. (1997). Adenovirus type 5 fiber knob binds to MHC class I alpha 2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO Journal* **16**, 2294–2306.

Karayan, L., Gay, B., Gerfaux, J. & Boulanger, P. A. (1994). Oligomerization of recombinant penton base of adenovirus type 2 and its assembly with fiber in baculovirus-infected cells. *Virology* **202**, 782–795.

Karayan, L., Hong, S. S., Gay, B., Tournier, J., Dupuy d'Angeac, A. & Boulanger, P. (1997). Structural and functional determinants in Adenovirus type 2 penton base recombinant protein. *Journal of Virology* **71**, 8678–8689.

Kidd, A. H., Chroboczek, J., Cusack, S. & Ruigrok, R. W. (1993). Adenovirus type 40 virions contain two distinct fibers. *Virology* **192**, 73–84.

Kieny, M. P., Lathe, R. & Lecocq, J. P. (1983). New versatile cloning and sequencing vectors based on bacteriophage M13. *Gene* **26**, 91–99.

Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T. & Curiel, D. T. (1996). Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *Journal of Virology* **70**, 6839–6846.

Mullis, K. G., Haltiwanger, R. S., Hart, G. W., Marchase, R. B. & Engler, J. E. (1990). Relative accessibility of *N*-acetylglucosamine in trimers of the adenovirus type 2 and 5 fiber proteins. *Journal of Virology* **64**, 5317–5323.

Novelli, A. & Boulanger, P. A. (1991). Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. *Virology* **185**, 365–376.

Pieniazek, N. J., Slemenda, S. B., Pieniazek, D., Velarde, J., Jr & Luftig, R. B. (1990). Human enteric adenovirus type 41 contains a second fiber protein gene. *Nucleic Acids Research* **18**, 1901.

Roelvink, P. W., Lizonova, A., Lee, J. G. M., Li, Y., Bergelson, J. M., Finberg, R. W., Brough, D. E., Kovesdi, I. & Wickham, T. J. (1998). The coxsackie–adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *Journal of Virology* **72**, 7909–7915.

Signas, C., Akusjarvi, G. & Pettersson, U. (1985). Adenovirus 3 fiber polypeptide, implications for the structure of the fiber protein. *Journal of Virology* **53**, 672–678.

Stevenson, S. C., Rollence, M., White, B., Weaver, L. & McClelland, A. (1995). Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *Journal of Virology* **69**, 2850–2857.

Svensson, U., Persson, R. & Everitt, E. (1981). Virus–receptor interaction in the adenovirus system. Identification of virion attachment proteins of the HeLa cell plasma membrane. *Journal of Virology* **38**, 70–81.

Tomko, R. P., Xu, R. & Philipson, L. (1997). HCAR and MCAR, the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proceedings of the National Academy of Sciences, USA* **94**, 3352–3356.

Wickham, T. J., Mathias, P., Cheresh, D. A. & Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**, 309–319.

Wickham, T. J., Filardo, E. J., Cheresh, D. A. & Nemerow, G. R. (1994). Integrin $\alpha v\beta 5$ selectively promotes adenovirus cell membrane permeability. *Journal Cell Biology* **127**, 257–264.

Xia, D., Henry, L. J., Gerard, R. D. & Desenhofer, J. (1994). Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1·7 A resolution. *Structure* **2**, 1259–1270.

Received 9 November 1998; Accepted 14 January 1999