

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 capsomers

Saw See Hong and Pierre Boulanger¹

Laboratoire de Virologie et Pathogénèse Moléculaires (CNRS URA 1487), Institut de Biologie, Faculté de Médecine, 34060 Montpellier, France

¹Corresponding author

A filamentous phage-displayed random hexapeptide library was screened on the adenovirus type 2 (Ad2) penton capsomer and its separate domains, penton base, full-length fiber, fiber shaft and fiber knob. Affinity supports were designed to immobilize the penton ligate with a preferred orientation, via immunoadsorption to pre-coated antibody. Three classes of phagotopes were distinguished in the eluates from the penton and fiber domains. (i) The first class represented peptide sequences identified in certain Ad2 capsid proteins, protein IIIa, protein pVIII, penton base and penton fiber. Data from specific ligand elution of phages bound to fiber and penton base wild-types and mutants suggested that the region overlapping the RLSNLLG motif at residues 254–260 in the penton base and the FNPVYP motif at residues 11–16 in the fiber tail formed mutual interacting sites in the penton capsomer. (ii) The second class consisted of phagotopes homologous to peptide sequences found in host cell membrane proteins involved in receptor or adhesion functions. One of the most abundant species corresponded to a conserved motif present in the β -strand B of type III modules of human fibronectin. In addition, phages which were screened for their failure to bind to penton base RGD mutants were found to carry consensus motifs to peptide sequences present in the RGD recognition site of human integrin β subunits. (iii) The third class comprised peptide motifs common to both viral and cellular proteins, suggesting that a mechanism of ligand exchange could occur during virus entry and uncoating, and virus assembly and release. *Keywords:* adenovirus/Ad2 receptor/fiber shaft/fiber knob/phagotopes

Introduction

The vertex capsomer of the adenovirus (Ad) icosahedral capsid, the penton, is a heteromeric protein composed of two different late gene products, the penton base subunit, anchored in the capsid, and a fibrous projection, the fiber (Wilcox and Ginsberg, 1963; Valentine and Pereira, 1965; Nermut, 1984; Pettersson, 1984). The Ad penton is composed of four major domains with different structural and functional roles, namely (i) penton base, (ii) fiber tail, (iii) fiber shaft and (iv) fiber knob. The tail, which comprises the N-terminal 40 residues in the serotype 2 fiber, has been shown to be the domain of binding of the fiber to

the penton base (Devaux *et al.*, 1987; Weber *et al.*, 1989; Novelli *et al.*, 1991b; Karayan *et al.*, 1994). The shaft sequence is characterized by a 15 residue repeat motif, of which the number varies according to the different Ad subgroup, resulting in various lengths for the fibers (Norrby, 1969; Green *et al.*, 1983; Signäs *et al.*, 1985; Ruigrok *et al.*, 1990; Stouten *et al.*, 1992; Kidd *et al.*, 1993). The distal knob domain comprises the C-terminal 180 residues of the fiber sequence, of which the last 40 have been found to be required for fiber trimerization (Novelli and Boulanger, 1991a,b).

The penton capsomer, which represents the most accessible and outermost structural component of the virion, owing to the fiber projection and its distal knob (Xia *et al.*, 1994), has been found to be essential for human adenovirus–host cell interactions and virus infection. Thus in the currently accepted model of sequential interactions between Ad and its cell receptor(s), the primary event would consist of virus–cell recognition and attachment, involving the fiber knob and an as yet unidentified cell surface receptor. Subsequently the conserved RGD motifs in the penton base and host cell components (such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins) within the plasma membrane would interact and permit (or facilitate) endocytosis of the virion (Lonberg-Holm and Philipson, 1969; Hennache and Boulanger, 1977; Svensson *et al.*, 1981; Neuman *et al.*, 1988; Defer *et al.*, 1990; Varga *et al.*, 1991; Bai *et al.*, 1993; Belin and Boulanger, 1993; White 1993; Wickham *et al.*, 1993; Cuzange *et al.*, 1994; Mathias *et al.*, 1994; Nemerow *et al.*, 1994; Sprengel *et al.*, 1994). However, significant differences in RGD ligand usage have been observed between Ad2 and Ad12 (Bai *et al.*, 1994) and additional capsid proteins, which remain to be identified, could also participate in Ad cell entry (Greber *et al.*, 1993; Wickham *et al.*, 1994). This is the case for proteins located at or near the vertices of the virus icosahedron, as suggested for peripentonal hexon-associated protein VI (van Oosterum and Burnett, 1985; Stewart *et al.*, 1991, 1993) or for the capsid protein IIIa (Everitt *et al.*, 1975; Boudin *et al.*, 1980; Cuillel *et al.*, 1990).

The aim of the present study was to investigate further the host cell membrane proteins involved in Ad2 attachment (referred to as ‘fiber receptors’) and Ad2 endocytosis (termed ‘penton base receptors’) and the possible subsets of membrane receptor peptide sequences interacting with the external components of the adenovirion. For this purpose we used the technique of affinity selection of complementary peptide ligands from a filamentous bacteriophage-displayed random peptide library on immobilized ligates (Parmley and Smith, 1988; Cwirla *et al.*, 1990; Scott and Smith, 1990). This technique has been successfully applied to the identification of (i) the epitope recognized by a monoclonal antibody (Stephen

and Lane, 1992; Yaron *et al.*, 1993), (ii) the immunofootprints of pathogens in human sera (Folgori *et al.*, 1994) and (iii) complementary peptide sequences in partner proteins of integrins (Koivunen *et al.*, 1993, 1994), cellular chaperones (Blond-Elguindi *et al.*, 1993) or concanavalin A (Oldenburg *et al.*, 1992; Scott *et al.*, 1992).

For this analysis we developed several strategies for the isolation and characterization of Ad capsid-specific hexapeptide phagotopes displayed in the pIII minor coat protein of fd-derived filamentous phage fUSE5 (Scott and Smith, 1990) from over 10⁸ different sequences. (i) Phage biopanning was performed using a variety of Ad penton-derived affinity systems. This included wild-type penton capsomers (base + fiber) isolated from Ad2-infected HeLa cells and immobilized on a solid support and each of its structural domains, namely penton base, full-length fiber, fiber shaft and fiber knob, expressed as recombinant proteins in baculovirus-infected insect cells (Novelli and Boulanger 1991b; Karayan *et al.*, 1994; Louis *et al.*, 1994). (ii) Penton base mutants carrying amino acid substitutions in the conserved RGD motif consensus to adhesion sequences were biopanned with the same library. (iii) Phage biopanning was also carried out on immobilized penton ligate specifically oriented through its binding to pre-coated polyclonal or monoclonal antibodies (mAb). (iv) Selective elution of phages by competition with an excess of specific viral ligand (Blond-Elguindi *et al.*, 1993; Smith and Scott, 1993), i.e. soluble fiber with immobilized penton base or soluble penton base with immobilized fiber, was also designed to probe the peptide sequences involved in penton base and fiber interactions.

The results of our phage biopanning revealed a complex picture for the ligands of the Ad2 penton capsomer. Comparison of our protein sequence data with those in gene banks suggested that they belonged to two major classes. One class of motifs corresponded to Ad2 capsid components, among which some have already been identified as penton partner proteins. The second class of peptide ligands were homologous to sequences found in cell membrane proteins with receptor or adhesion functions. Certain sequences were found to be common to these two classes.

Results

Identification of mAb epitopes by phage biopanning

In order to validate our affinity systems, as well as to characterize the immunological probes used in our study, the epitopes of two different mAb were characterized by biopanning of the fUSE5 phage-displayed hexapeptide library. The first mAb was directed against the fiber (4D2.5), the second was against the penton base (20G1). MAb 4D2.5 recognizes an epitope which is conserved in Ad2 and Ad5 fiber, reacts better with SDS-denatured fiber monomer than intact trimer in immunoblotting and has been localized within the N-terminal tail by peptide mapping (Hong and Engler, 1991). This was further supported by an absence of reactivity to our N-terminal truncated fiber mutant FiAT17 (Novelli and Boulanger, 1991b). The hexapeptide sequences biopanned on mAb 4D2.5 are presented in Table I. Among the phagotopes isolated the central tetrapeptide core NPVY was con-

Table I. Phagotopes biopanned on anti-fiber and anti-penton base monoclonal antibodies^a

Anti-fiber tail mAb 4D2.5	Anti-penton base mAb 20G1	Background (BSA)
FNPVYP	RQFQSP	SLERSI
QFNVPY	RQNQSP	KQVKLN
FNPAYY	SQFQSP	VWGVFP
MNPAYS	SQFQAP	SLRWGS
MNPVYS	SQFQAP	DLRSAF
MFNPVS	IQFQGP	IARRAI
MFNPVS	FQWQGP	RVIPMY
MFNPVS	QWQAPA	FIPLFS
MFNPVS	QWQAPA	RFIWRW
YFNPAY	QGQAPV	REMFVY
AYNPIY		FLMVRT
YNWVYD		
Consensus	Consensus	
FNPVYP	RQ(W,F)Q(S,A)P	
Fiber sequence	Penton base sequence	
¹⁰ TFNPVYPYD ¹⁸	²⁶⁴ RQPFQE ²⁶⁹	

^aThe phage-displayed hexapeptide library was biopanned on immobilized mAb. Colonies of Tet + Kan-resistant bacterial cells, corresponding to mAb-bound phages, were isolated and their phagotome-harboring recombinant pIII genes were sequenced, as described in Materials and methods. Phages non-specifically adsorbed onto bovine serum albumin-coated microtitration wells showed some samples of the background binding.

sistently found, with some variations observed at the first and last positions. F and Y were found at higher frequencies as the first residue and P was found once as the last one. A similar peptide motif, FNPVYP, is present between residues 11 and 16 in both Ad2 and Ad5 and was found to be highly conserved within the fibers of other serotypes (Kidd *et al.*, 1993). This confirmed the previous mapping of 4D2.5 and its identification as a non-conformation-dependent, linear epitope.

The anti-penton base mAb 20G1 had been selected against recombinant penton base isolated in its native form. Preliminary characterization by immunoblotting assays had shown that 20G1 reacted with non-denatured pentamers and monomers of wild-type penton base and N-terminal deletion mutant PbAT69 and C-terminal deletion mutant PbCT531, suggesting that its epitope was localized in the central domain of the penton base, within residues 70–530 (Karayan *et al.*, 1994). The 20G1 epitope was further characterized by phage library biopanning (Table I). The consensus phagotome found was RQXQZP, in which X was an aromatic residue (phenylalanine or tryptophan) and Z an amino acid residue with a short side chain (serine or alanine). The best fitting peptide sequence in the Ad2 penton base protein was the RQPFQE motif at position 264 (Roberts *et al.*, 1986), in a region presenting a high probability of accessibility and antigenicity.

Design of affinity supports with specific orientation of the ligates

Adenovirus apex-mimicking affinity support. The penton capsomer is an asymmetrical molecule with a well-defined polarity at each of the 12 vertices of the virion. The penton base constitutes an integral part of the capsid, whereas its fiber projection, which terminates in a distal knob, gives the isolated penton capsomer the aspect of a

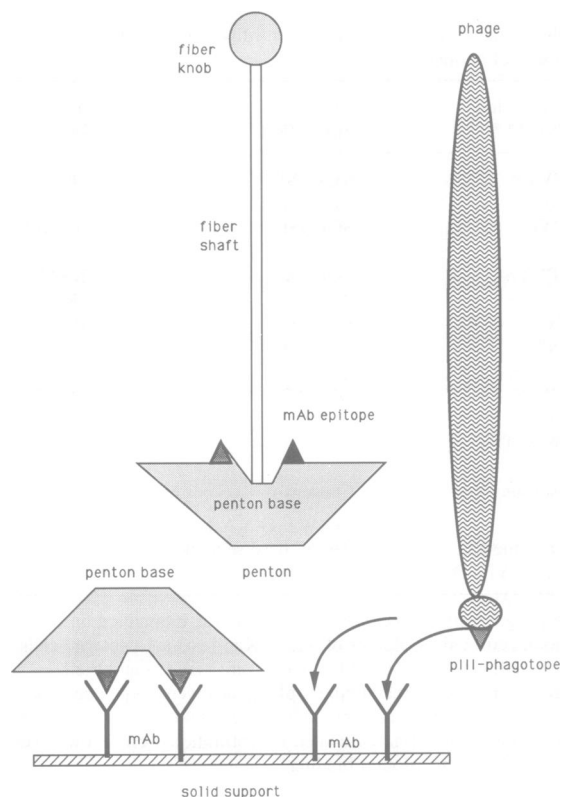


Fig. 1. Phage panning on an affinity support coated with mAb. In the case depicted here a mAb directed against the penton base was pre-coated onto a microtitration plate before adsorption of Ad penton capsomer. If the mAb epitope is in the vicinity of the fiber attachment site (e.g. mAb 20G1) steric hindrance will prevent adsorption of intact penton onto the support and only free penton base would be capable of binding to the mAb-coated support. The antigen binding domain of the mAb will then be available for phage binding and the phagotome motif isolated will represent the mAb epitope. Note that the different components are not represented to their respective scale.

dumb-bell shaped structure under the electron microscope (Pettersson, 1984; Ruigrok *et al.*, 1990; Karayan *et al.*, 1994). It has been shown that the N-terminus of the fiber polypeptide chain is engaged in binding with the penton base, whereas its C-terminus forms the terminal knob (Devaux *et al.*, 1987; Weber *et al.*, 1989). To screen for host cell peptide sequences which recognize the Ad2 virion it was therefore essential in our biopanning assays to mimic the natural orientation of the penton capsomer, as in the capsid. This was achieved by pre-coating the affinity support with polyclonal antibody against the penton base, followed by immunoadsorption of penton capsomer, so as to have the penton anchored via its base and with its distal fiber knob oriented upwards. The advantage of such an oriented ligate was to ensure that reactive peptide sequences on the external domains of the penton capsomer would be accessible to the phages for binding, as occurs in the virion during host cell recognition.

Affinity support with monoclonal antibody-oriented penton. Alternatively, the orientation of the penton ligate was directed via its specific binding to a pre-coated penton base mAb, of which the epitope has been identified in the sequence. As depicted in Figure 1, if the penton base mAb epitope is at or close to the fiber binding site, the epitope would be inaccessible in the complete penton

capsomer by steric hindrance. In this case only free penton base, if any, would be able to adsorb to the affinity support. In the absence of saturating amounts of free penton base in the ligand protein sample, free Fab would be available to select phagotopes corresponding to the mAb epitope. On the other hand, if the mAb epitope is far from the fiber binding site in the penton base three-dimensional structure, complete penton capsomer (namely base + fiber) would be retained on the pre-coated mAb layer and no Fab would be available after saturation by penton.

Therefore, phage biopanning on affinity supports with mAb-oriented ligates represents an indirect method to determine the topology of the fiber binding site at the surface of the penton base capsomer with respect to a mAb with defined epitope. As shown below, we used both these indirect approaches and the ligand elution method to identify the fiber binding site in the penton base.

Biopanning of the hexapeptide library on penton and penton base capsomers

Ad2 penton was immobilized by adsorption to pre-coated penton base antibody, biopanned with the recombinant phage library and the bound phages eluted at low pH. The phagotopes isolated were sorted into different groups, according to the respective position of the amino acid residues, the charge, hydrophathy and bulk of their side chains. Five groups were thus defined for the Ad2 penton and each group identified by a consensus motif which was the most represented sequence (Table II). For example, groups I (GLSIWN), II (NGSYPL) and III (AQQHYA) were composed of homogeneous and highly represented phagotopes. Members of groups IV (NDMISR) and V (TLR) were more heterogeneous, as each individual sequence was represented by only one or a few copies. The GLSIWN, NGSYPL and AQQHYA motifs were also eluted from the penton base, but three phagotome groups, VI (MTSDDL), VII (VEPATP) and VIII (RWYRPI), were unique to the penton base and were not found in the acid eluate from penton adsorbed via its penton base moiety (Table II).

Biopanning of the hexapeptide library on the full-length fiber and fiber domains

Three motifs, GLSIWN (group I), AQQHYA (group III) and NDMISR (group IV), which were found previously in the penton and penton base eluates, were also recovered from full-length fiber, but their respective groups showed more sequence heterogeneity (Table III). This could be due to a random adsorption of fiber onto the surface and/or, alternatively, to some heterogeneity in the fiber quaternary structure. Both the recombinant full-length fiber and fiber knob have been shown to exist as true trimers (Henry *et al.*, 1994; Louis *et al.*, 1994), whereas fiber shaft expressed by FiDT61-410 (residues 61–410 in the Ad2 fiber sequence) has been shown to occur mainly in monomeric and dimeric forms (Novelli and Boulanger, 1991b).

Biopanning of the phage library on the separate fiber domains, shaft and knob, also yielded phagotopes homologous to the GLSIWN motif, but AQQHYA-related peptides were only found with full-length fiber trimer and members of the NDMISR group were only found with the shaft. This would suggest that both NDMISR and

Table II. Hexapeptide phagotopes eluted from penton capsomer (base-fiber complex isolated from Ad2-infected HeLa cells) and from recombinant penton base (pb)^a

Group	Penton (low pH eluted)	Recombinant pb (low pH eluted)	Recombinant pb (fl-fiber eluted)
(I)	GLSIWN	GLSIWN	GLSIWN
	GLSIWN	GLSIWN	GLSIWN
	GLSIWN	GLSIWN	GLSIWN
	GLSIWN	GLSIWN	GLSIWN
	GLSIWN	GLSIWN	GLSIWN
	GLSIWN	LGEWSF	GLSIWN
	GLSIWN	GRLWDT	
	GLSIWN	WDAVNL	
	FESIWP	WKWVNL	
	WDGLSP	HSWLSH	
	PVWLSQ ^b	ASPWLV APWKVW	
(II)	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
	NGSYPL	NGSYPL	NGSYPL
(III)	AQQHYA	ASQSHF	GQQDYA
	AQQHYA	AQFQHH	GQQDYA
	AQQIML	GITHVA QLPAKQ HQLDSA	GQQDYA
(IV)	NDMISR		
	NDMISR		
	NDMISR		
	IDLMSI		
	RTKMSL HMTRND		
(V)	TNSLRA	MNTLRY	
	LTARSI	RMNTKH	
	ATSHRI	NRMRYL	
	TERLHL	NSQRYK	
		AEKLRV	
		YRSKSS	
		YVKSRF	
		FYTLHT	
(VI)		MTSDDL	
		SMDADD	
		DDTYLF LDLDPG	
(VII)		VEPATP	
		VEPATP	
		YIRVEP	
		EYTPDR ^b SRPDQR ^b	
(VIII)		RWYRPI	
		RWYRPI	
		RWYSPI	
		RTYRPI	
		RAAWPI KAIWRW	

^aPhages were adsorbed on the immobilized penton, isolated from Ad2-infected HeLa cells or on recombinant penton base (pb), expressed in baculovirus-infected Sf9 cells, as described in Materials and methods. Elution was carried out using low pH buffer (total elution) or, more specifically, by competition with the natural ligand of penton base, soluble wild-type full-length fiber (fl-fiber).

^bReverse orientation

Table III. Hexapeptide phagotopes eluted from recombinant full-length (fl) fiber and from its separate domains, shaft and knob^a

Group	fl-fiber (acid eluted)	Fiber knob (acid eluted)	Fiber shaft (acid eluted)	fl-fiber (pb eluted)
(I)	GLSIWN	TRVSWE	IRVRTW	
	MVLFWS	WSIAVS ^b	LVRQVF	
	TLSVFG	FSWLVI ^b		
	KWDTRR	TIFITI		
	WWVIRD ^b			
(III)	AQQHYA			
	NTQYQL			
	AVYHGQ ^b			
(IV)	NDMISR		MRLTNM	
	VLENSM		GLRMFM	
	FSTNGM			
	RLMGVI ^b			
(IX)	MPKSLR	PKLGNH		
	PQLVPS	PVRHPN		
	IGLPTR	MPAHPF		
		PHFARS		
		SPRNKF RTPTLF		
(X)	RARRSI	LPRARA		
	RIRLEN	RIRSLI		
(XI)	KKCCYI	KKCCYI	KKCCYI	
		KKCCYI	KCCYST	
			KCCYST	
			KCCFYV	
(XII)	GRIFRH	STLVRY	AGIFRH	
	GRIFRH	YRVLRT	VRSHLL	
	TRLYDR	THSKSF	HKTGLF	
	DRRYMP	SHHSSQ	TEHRID	
			VLTVIH	
(XIII)		GAFFIG	GGFELI	
		GAILVI	GALLFP	
		KNLFFI		
		SSRVFF		
		LSAGLA		
(XIV)				RRVLLG
				RRVLLG
				RRVLLG
				RRVLLG
				RRVLLG
				RRVLLG
			RYVLPV	

^aPhages were adsorbed on recombinant fiber proteins isolated from baculovirus-infected Sf9 cells. Elution was carried out using low pH buffer (total elution) or, more specifically, by competition with the natural ligand of the fiber, i.e. soluble penton base (pb), also obtained as recombinant protein. Refer to Materials and methods for details.

^bReverse orientation.

AQQHYA phagotopes were specific for the fiber shaft, but their binding would depend upon the conformational structure of the fiber ligate: AQQHYA would preferentially recognize the fiber shaft in its trimeric structure, as in the full-length recombinant fiber FiFL582, whereas the monomeric and dimeric forms of the fiber shaft expressed by the double truncated mutant FiDT61-410 would be

recognized by NDMISR and its variants. Phagotopes of group IX, typified by the PKLGNH-HPN motif, were among the most represented species in the fiber knob eluate (Table III). For the fiber shaft the most homogeneous group (group XI), represented by the KKCCYI motif, was also found in the full-length fiber and knob eluates, suggesting that its binding did not depend on fiber quaternary structure. Since the amino acid sequence between residues 388 in the shaft and 410 in the knob was common to the two fiber constructs FiAT388 and FiDT61-410, the KKCCYI phagotope could recognize the shaft-knob junction, which contains the highly conserved TLWT(X)₃P motif (Kidd *et al.*, 1993). This could also be the case for members of group XIII (Table III).

Phage displacement using specific adenoviral ligands

Acid elution of phages from an immobilized protein ligate theoretically yielded the whole bulk of bound phagotopes. In contrast, in ligand elution assays, in which phages were displaced by competition with a natural ligand of the immobilized protein, the eluate would be expected to contain a more restricted population of phagotopes. In the case of the penton base as the immobilized ligate the natural ligand was the soluble fiber and, vice versa, soluble penton base was the ligand for the immobilized fiber. Considering the immobilized penton base, there were three possible mechanisms of ligand elution using soluble fiber, as depicted in Figure 2. (i) Phage species carrying a hexapeptide motif complementary and adapted to the peptide sequence of the fiber attachment site in the penton base would be displaced by an excess of soluble fiber, via direct competition for the same binding site. The isolated phagotope should therefore mimic the motif in the fiber which recognized its natural binding site in the penton base. (ii) Phages harboring hexapeptides recognizing sites in the penton base molecule other than, or distant from, the fiber binding site, could be released from their ligate as a result of conformational changes occurring upon fiber attachment and subsequent decrease, or loss, of affinity for their own ligands. This mechanism would be analogous to that observed with allosteric sites of allosteric enzymes. (iii) The third mechanism would yield phages adsorbed to the immobilized penton base via phagotopes complementary to sequence(s) or motif(s) also present in the soluble fiber ligand. If both viral proteins carry similar binding site(s), phage-displayed hexapeptide(s) would be displaced from the immobilized ligate by an excess of soluble ligand. The latter two mechanisms are not mutually exclusive, since phages which were released from an allosteric-like site in the penton base upon fiber attachment could in turn find a similar binding site available on soluble fiber molecules and be recovered in the supernatant.

Ligand elution of phages bound to the penton base yielded three homogeneous populations of phagotopes (Table II), carrying the GLSIWN, NGSYPL and GQQDYA motifs respectively. The third mechanism most likely applied to two of these phagotope motifs, GLSIWN and GQQDYA, since they were found in the acid eluates from both penton base and fiber ligates. Thus the existence of common (or homologous) accessible peptide sequences in the penton base and fiber could be hypothesized (Tables II and III). For example, the peptide motif LDVD is found

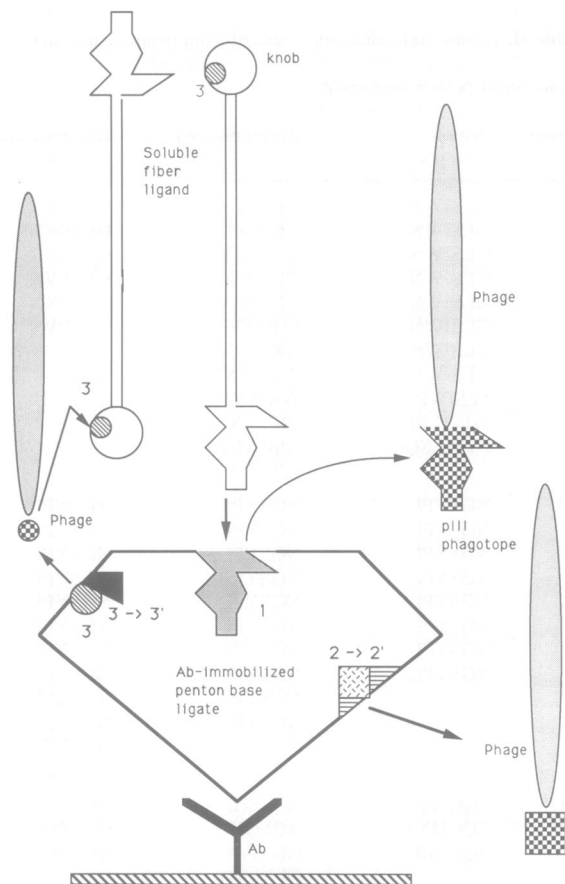


Fig. 2. Theoretical mechanisms of phage displacement by using specific ligand elution. In the example sketched the penton base represents the immobilized ligate, adsorbed to the solid support via anti-penton base Ab, whilst soluble fiber is used as the competing ligand. (1) Direct competition between fiber and phage occupying the fiber binding site of the penton base. (2) Conformational change in the phage binding site (allosteric-like site) and decrease in its affinity for the phage upon fiber attachment. (3) Displacement of the equilibrium between bound and unbound phages by an excess of soluble fiber ligand providing extra binding sites in its own sequence. (3') A combination of (2) and (3), in which the binding of the fiber to its natural site provokes a change in the conformation of another site (3→3'). After release from the penton base the phage could in turn bind to an equivalent site available on the soluble fiber. Note that the diagram is not drawn to scale.

at positions 287–290 in the penton base and at positions 268–271 in the fiber shaft (Roberts *et al.*, 1986). This hypothesis was further investigated using fiber N-terminal mutant FiAT61. Deletion of the fiber tail domain in recombinant fiber mutant FiAT61 abolished its binding to the penton base *in vivo* (Karayan *et al.*, 1994). Equivalent input multiplicities of the three phages species, GLSIWN, GQQDYA and NGSYPL were pooled, co-adsorbed onto immobilized wild-type penton base and analyzed by ligand elution assays using FiAT61. GLSIWN and GQQDYA motifs represented 85–90% of the phagotopes found in the eluates, confirming that competition with these two phages occurred in the absence of binding of the fiber to the penton base. The NGSYPL phagotope, however, which was only found in eluates from the penton and penton base and never from the fiber ligate (Tables II and III), was not competed away in significant amounts by the penton base binding-defective fiber mutant FiAT61 (10–

15%). This suggested that its complementary sequence was only present in the penton base and that the NGSYPL phagotopelution proceeded by fiber competition via the first or second mechanism.

When fiber was used as the immobilized ligate for biopanning and penton base as the soluble ligand a homogeneous population of recombinant phages were eluted, with the preferred hexapeptide motif RRVLLG (Table III). Since this sequence was unique to the biopanned fiber and was never found among the phagotopes isolated with the penton base, it was assumed to be eluted from the fiber by direct competition with soluble penton base for the same complementary peptide domain (first mechanism described above). The RRVLLG motif, consensus to the RLSNLLG peptide in the Ad2 penton base sequence at position 254 (Roberts *et al.*, 1986), would therefore correspond to the fiber binding site in the penton base protein. The following experiments were designed to test this hypothesis.

Phage adsorption assay with fiber and penton base mutants

The RRVLLG-harboring phage was amplified and adsorbed on microplate wells coated with the recombinant full-length fiber FiFL582, the two N-terminal-truncated mutants FiAT17 and FiAT61 and the recombinant fiber knob FiAT388. All these recombinant fiber gene products occur as trimers (Novelli and Boulanger, 1991b; Louis *et al.*, 1994). Phages were eluted using the penton base as the soluble competitor and infectious titers determined in the eluates. As shown in Table IV, there was a significant difference in the fiber binding capacity of the RRVLLG-containing phages between wild-type fiber and fiber mutants carrying N-terminal deletions. This confirmed the role of the RRVLLG-RLSNLLG motif in the interaction of the penton base with the fiber tail domain.

Likewise, since the RGD motif in the penton base has been implicated in Ad endocytosis, a similar phage adsorption assay was used in an attempt to identify possible RGD recognition sequence(s) among the variety of phagotopes retained on recombinant penton base (Table II). A population of 96 individual phages, which were first selected by biopanning on recombinant penton base wild-type, were assayed for their binding capacity to penton base wild-type and two penton base mutants carrying non-conservative amino acid substitutions in the RGD motif at position 340, PbR340E and PbR340G. Phages which consistently showed lower titers after micropanning on both RGD mutants were isolated and their recombinant pIII protein sequenced. Two recombinant phages, harboring the sequences MTSDDL and SMDADD respectively, were thus identified. The binding capacity of the MTSDDL-containing phage to the penton base wild-type and the PbR340E and PbR340G mutants are shown in Table IV.

Biopanning of the hexapeptide library onto mAb-oriented penton ligate

When the affinity binding system consisted of the immobilized penton adsorbed onto pre-coated anti-penton base mAb 20G1 the majority of the phagotopes sequenced belonged to the (S,R)Q(F,W)QAP species, even after multiple and prolonged saturation steps of the mAb with

Table IV. Microtitration of RRVLLG and MTSDDL phages adsorbed on wild-type and mutant recombinant fiber and penton base^a

Immobilized ligate	No. of colonies	
	RRVLLG ^b	MTSDDL ^c
FiFL582 (full-length, wild-type)	21.9 ± 9.6	
FiAT17 (N-terminal deleted)	6.0 ± 1.5	
FiAT61 (tail-deleted)	6.6 ± 2.3	
FiAT388 (knob)	4.4 ± 2.3	
Control BSA	2.3 ± 1.1	
PbFL571 (full-length, wild-type)		62.2 ± 16.0
PbR340E (RGD mutant)		4.2 ± 2.2
PbR340G (RGD mutant)		8.3 ± 4.8
Control BSA		2.1 ± 1.5

^aAliquots (50 µl) from a suspension of recombinant fUSE5 phages harboring the RRVLLG or MTSDDL hexapeptide and with an input titer of 10⁵ phages/ml were adsorbed on immobilized recombinant fiber or penton base proteins and their mutants. After extensive rinsing the phages were eluted and the infectious titers determined by colony titration as described under Materials and methods. Values in the table are mean ± confidence interval at the *P* = 0.05 level (*n* = 12). The number of phages adsorbed on full-length fiber or wild-type penton base corresponded to 40–45% of the input phages, versus 5–10% for the control BSA-coated wells.

^bElution by competition with soluble ligand, using wild-type recombinant penton base as the specific ligand of the fiber.

^cLow pH elution of MTSDDL phages.

a penton sample (data not shown). This strongly suggested that the anti-penton base mAb 20G1 was incapable of binding to intact penton capsomer. This was confirmed by co-immunoprecipitation assays of fiber-penton base complex using mAb 20G1. Unlike polyclonal antibody (Karayan *et al.*, 1994), mAb 20G1 failed to co-precipitate the fiber and penton base from lysates of Sf9 cells co-infected with penton base- and fiber-expressing recombinants and only free penton base was detected in 20G1 immunoprecipitates (data not shown). This would imply that access of mAb 20G1 to its epitope was blocked, due to steric hindrance, by attachment of the fiber to the penton base (Figure 1) and that both sites were likely to be close to each other in the penton base three-dimensional structure. Indeed, the RQPFQ motif, homologous to the 20G1 epitope, occurs at position 264 in the penton base, in close vicinity to the RLSNLLG motif at position 254, which was identified as a fiber binding site (Tables III and IV).

Phage interference with penton capsomer assembly

Three phage clones were assayed for their possible inhibitory effect on penton base and fiber assembly *in vitro*. Two phagotopes had been identified as candidates for mimicking the mutual interacting sites of the penton base and fiber: (i) NGSYPL, isolated by biopanning and ligand eluted from the penton base (Table II); (ii) RRVLLG, from the fiber (Table III). (iii) A third, FNPVYP, corresponded to a linear epitope of the fiber tail (Table I) in a region which has been shown to be masked when anchored to the penton base (Devaux *et al.*, 1987; Weber *et al.*, 1989). As shown in Figure 3, the three phages competed with penton base-fiber assembly in a dose-dependent manner. RRVLLG showed a slightly higher inhibition efficiency than the two other phages, with a 50% inhibitory dose of ~10⁹ phage virions/sample, versus 10¹⁰ for both NGSYPL

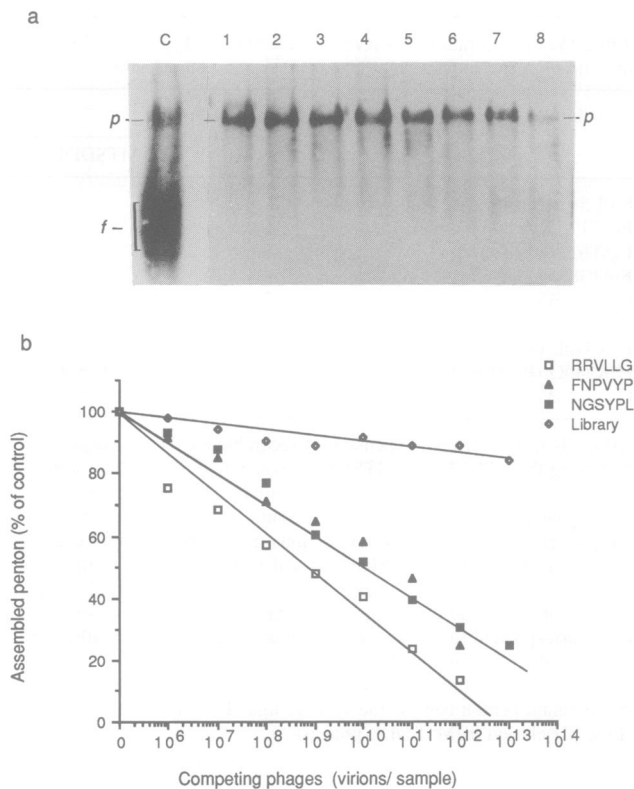


Fig. 3. Phage interference with penton capsomer assembly *in vitro*. Recombinant penton base PbFL571 was incubated with recombinant fiber FiFL582 in the presence of increasing amounts of competing phages, ranging from 10⁶ (lane 2) to 10¹² (lane 8) virions/sample. Control samples are shown in lanes C and 1. C, Ad2 soluble fiber and penton; 1, no phage added. The efficiency of penton assembly was estimated using the band mobility shift assay. Upon assembly with the penton base, free fiber protein (diffuse band *f*) is retarded and migrates as a discrete band of penton (*p*) with a lower electrophoretic mobility in the gel and reacts with both fiber and penton base antibodies. (a) Luminescent immunoblot pattern of the assembly competition assay with RRVLLG phage, as revealed by fiber antibody. (b) Densitometric scanning analysis of the penton bands as presented in (a). The results (average of four separate experiments) were expressed as a percentage of the penton band signal found in control samples; SD were within 10–15% of the mean values.

and FNPVYP. The physiological significance of this difference with respect to the penton assembly process is uncertain, but this could be due to the stoichiometry of the penton capsomer subunits, in which three fiber subunits interact with five penton base subunits (van Oostrum *et al.*, 1987).

Effect of phages on Ad2 cell attachment and entry

The adsorption to plasma membrane receptors of ¹⁴C-labeled Ad2 virions was assayed at 4°C and virus entry into HeLa cells at 37°C in the presence of some representative clones of phages isolated by biopanning on the fiber (LPRARA), the penton base (MTSDDL) or both (GLSIWN), and used as competitors in single phage or multiple phage competition assays. Cell monolayers were incubated with constant, saturating amounts of radiolabeled Ad2 virions representing a 10-fold excess over the maximum theoretical number of surface receptors and the number of competing phage virions added per sample corresponded to 1-, 2-, 20- and 2000-fold the number of cell receptors respectively. Only a discrete competition

effect on Ad2 adsorption (20–25% decrease) was detected for a phage:receptor ratio of 2000 (data not shown), even when the three phages were added together and at the same final competing ratio. Since some degree of competition could be masked by co-adsorption of Ad2 and phage, via non-specific binding of phage to the HeLa cell surface or via an adenoviral bridge between cell and phage, Ad2 cell attachment was also assayed in the presence of DOC-solubilized phage pIII protein. No detectable increase in the level of inhibition of Ad2 attachment at low temperature was observed with recombinant protein pIII compared with phage virions (not shown). At 37°C, however, the total Ad2 cellular uptake was reduced to 2- to 3-fold the level of the control samples at a phage:receptor ratio of 2000 (Figure 4a) and the amounts of Ad2 label were also found to be reduced to similar levels (2- to 4-fold) in both the endosomal and nuclear compartments (Figure 4b and c). No significant difference was observed between the three different phages or a mixture of the three; the combined set of the three phages MTSDDL, LPRARA and GLSIWN did not result in any more pronounced effect. The observed inhibiting effect on Ad2 entry by phages was significant, considering that the maximum decrease in virus binding to HeLa cells obtained was 4-fold in homologous competition between radiolabeled Ad2 and a 100-fold excess of cold Ad2 competitor (Belin and Boulanger, 1993).

Relative affinities between phages and ligands

A collection of phages, isolated by biopanning on the penton base, the fiber or both, were immobilized on a solid support and analyzed with respect to their affinity for their viral ligands. The apparent association constants obtained (K_{app}), as derived from Scatchard plots of the binding reactions, ranged between 1 per μ M, for HSWLSH phage and the penton base, and 60 per μ M, for LGEWSF phage and the fiber (Table V). The relative affinities of phagotopes for their specific ligands seemed to vary according to the modifications from the consensus motifs. Thus the phage carrying the FNPVYP motif, identified as a linear portion of the fiber tail sequence recognized by mAb 4D2.5 (Table I), showed the highest K_{app} for the penton base, whereas the NGSYPL motif, which shared homology with the former but differed from the fiber linear sequence, showed a slightly lower affinity for the penton base ligand. Likewise, the MTSDDL phagotope, which failed to bind at significant levels to the RGD penton base mutant R340E, was found to have a higher affinity for wild-type penton base than phagotopes DDTYLF and SMDADD. MTSDDL has a higher sequence homology to the SMKDDLW motif present in the RGD binding site of the integrin β subunit (D'Souza *et al.*, 1988, 1994) than DDTYLF and SMDADD. However, there was no apparent relationship between the abundance of certain phagotopes isolated and their respective affinities for their ligands. For example, one of the most frequently isolated phage, which carried the GLSIWN hexapeptide, had a lower affinity for the penton base than the less commonly found LGEWSF (Tables I and V). This could reflect a difference in the number or/and accessibility of their respective complementary motifs in the immobilized viral ligate.

The relative affinities of the recombinant penton base

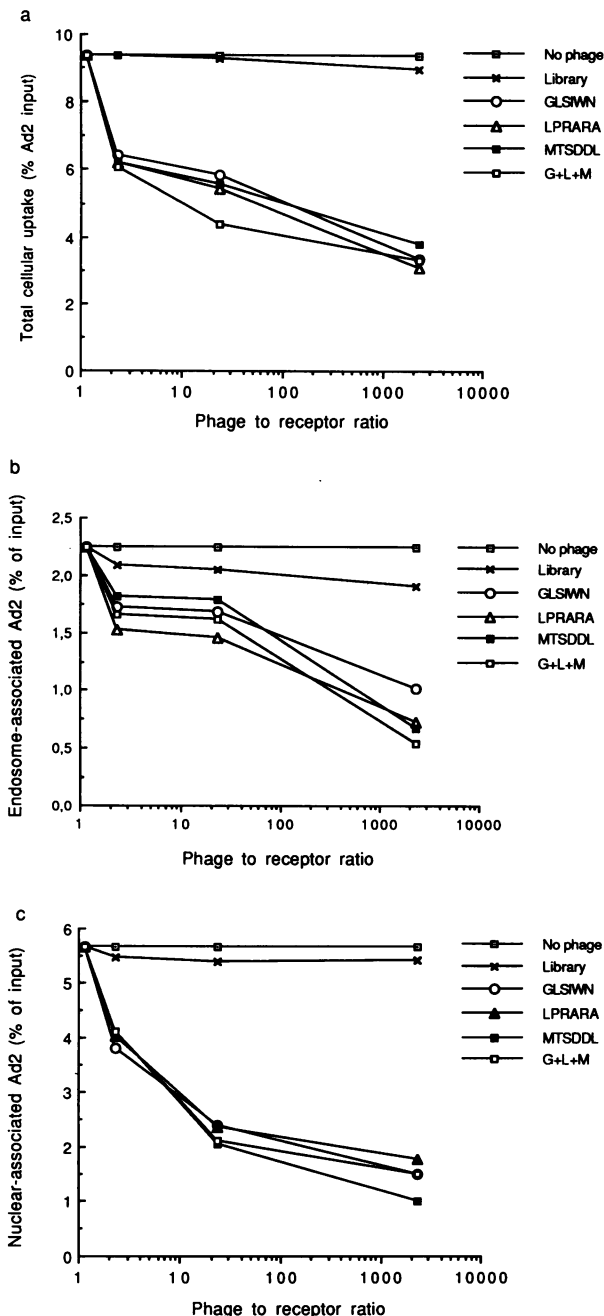


Fig. 4. Phage interference with Ad2 entry into HeLa cells. Cell monolayers were incubated at 37°C with constant, saturating amounts of [¹⁴C]valine-labeled Ad2 and increasing inputs of competing phages. Ad2 inoculum represented a 10-fold excess over the maximum theoretical number of surface receptors (10^4 receptors/cell, 5×10^9 receptors/well, 5×10^{10} Ad2 virions/well) and the number of competing phage virions added per sample corresponded to 1-, 2-, 20- and 2000-fold the number of cell receptors respectively. Competition assays were performed with three phages (GLSIWN, LPRARA or MTSDDL) individually or with a mixture of the three (G+L+M) and the phage starting library was used as a control. The quantity of intracellular Ad2 was determined from radioactivity in the whole cell pellets (a) and the endosomal (b) and nuclear (c) fractions respectively. The results (average of three separate experiments) were expressed as the percentage of total Ad2 input radioactivity (3×10^4 – 5×10^4 c.p.m./sample); SD were within 10–15% of the mean values.

Table V. Apparent association constants (K_{app}) of *in vitro* binding reactions between various immobilized hexapeptide-displaying phages and their soluble Ad2 protein ligands, recombinant wild-type penton base (r-pbase), recombinant wild-type fiber (r-fiber) and recombinant penton base mutant R340E^a

Phage hexapeptide or ligate	Labeled ligand	K_{app} (\pm SD) (per μ M)
Library ^b	r-pbase	≤ 0.1
FNPVYP	r-pbase	52.1 ± 11.5
NGSYPL	r-pbase	20.8 ± 8.2
GLSIWN	r-pbase	16.0 ± 1.9
MTSDDL	r-pbase	12.9 ± 2.0
DDTYLF	r-pbase	7.3 ± 4.0
SMDADD	r-pbase	6.4 ± 2.2
HSWLSH	r-pbase	1.0 ± 1.0
MTSDDL	Mutant R340E	≤ 0.5
Library ^b	r-fiber	≤ 0.1
LGESWF	r-fiber	64.7 ± 4.8
IRVRTW	r-fiber	52.7 ± 2.3
GLSIWN	r-fiber	18.5 ± 5.5
LPRARA	r-fiber	16.6 ± 5.7
RRVLLG	r-fiber	9.0 ± 3.0
Ad2 penton base ^c	Ad2 fiber	5.0 ± 3.0
HeLa cell ^d	r-fiber	476.0 ± 192.6
HeLa cell ^d	r-pbase	27.5 ± 5.0
KB cell ^e	Ad2virion	8.0 – 9.2×10^3

^aThe K_{app} were expressed as per ligand molarity, penton base, fiber or Ad2 virion respectively. The data presented in the table are the average of three separate experiments \pm SD.

^bThe penton base and fiber were found to bind to an immobilized total random phage library with a K_{app} of $< 10^5$ per M.

^cA dissociation constant of $K_d = 2 \times 10^{-7}$ M, expressed as fiber molarity, has been reported for the *in vitro* assembly reaction between the penton base and fiber isolated from Ad2-infected KB cells (Boudin and Boulanger, 1982), hence $K_{app} = 0.5 \times 10^7$ per M.

^dOur unpublished data. Dissociation constants of binding reactions with HeLa cell receptors were thus $K_d = 2$ nM for the Ad2 fiber and $K_d = 36$ nM for the penton base respectively. The values determined by Wickham *et al.* (1993) were 1.7 and 55 nM respectively.

^eData from Defer *et al.* (1990).

for most of its phagotopes were in the same range of values as for its cell receptor (Wickham *et al.*, 1993). For recombinant fiber, however, K_{app} was one order of magnitude higher for its cell receptor than for any of its phagotopes, suggesting that multiple discrete binding motifs contribute to the fiber binding site at the cell surface (Table V). The apparent association constant for the assembly reaction between Ad2 penton base and fiber *in vitro* has been previously estimated to be 5 per μ M (Boudin and Boulanger, 1982). A similar value (9 per μ M) was determined for the binding reaction of soluble recombinant fiber and immobilized phage carrying the RRVLLG motif, homologous to the penton base sequence RLSNLLG.

Discussion

In spite of the potential use of adenovirus in gene therapy, the mechanisms involved in viral attachment and subsequent cellular uptake remain poorly understood at the molecular level. The adenovirus particles enter their susceptible host cells by means of receptor-mediated endocytosis and it is well established that the fiber and penton capsomers are responsible for attachment of the virus at the cell surface (reviewed in Seth *et al.*, 1986; White, 1993; Nemerow *et al.*, 1994). The present study was

Table VI. Summation of the characteristic hexapeptide ligands of the penton, penton base (pb), full-length (fl) fiber, fiber knob and shaft domains^a

Class	Phagotope	Binding to	Homologous to or occurring in	
(i)	AQQHYA	Penton base	Ad2 IIIa (¹⁵⁶ GQEDYT) and Ad2 PrVIII (²³ AAQDY)	
	GQQDYA	fl-fiber		
	NDMISR	fl-fiber, shaft	Ad2 PrVIII (⁴⁰ MISR)	
	RRVLLG ^b	fl-fiber (pb-ligand eluted)	Ad2 pb (²⁵⁴ RLSNLLG)	
(ii)	GLSIWN	Penton base and fl-fiber	Human FN type III modules (SLLVSWQ, GVLTVSWE, TMRVTW, TVLVRW, etc.)	
	TRVSWE	Fiber knob,		
	IRVRTW	Fiber shaft		
	RWYRPI	Penton base		FN-receptor α -subunit (YRGRPI)
	MTSDDL ^c	Penton base		Human integrin β -subunit RGD-crosslinked site (SMKDDL)
	SMDADD	Penton base		
	VEPATP	Penton base		EGF receptor (VEPLTP)
	MNTLRY	Penton base		Human ankyrins (MNYLRY)
	RMNTKH	Penton base		Human membrane-associated folate-binding protein (CMNAKH)
	TRLYDR	fl-fiber		Human cadherins and annexins (QRLFDR)
	PQLVPS	fl-fiber		Human ICAM-1 (PQLVSP)
	MPAHPF	Fiber knob		Human LDL receptor (RLAHPF)
	LPRARA	Fiber knob		FN type III module 14 (PRARI), FN-III module 5 (PRAQI) and rat erythrocyte band-3 protein (SPRARAS)
	KLGNHPN	Fiber knob		Human cell receptor tyrosine kinases, cytoplasmic TK domain (e.g. KLGHHPN in <i>tie</i>)
	(iii)	KKCCYI		fl-fiber, knob and shaft
KCCFYV		fl-fiber, knob and shaft		
THSKSF		Fiber knob	Human C5a receptor (RESKSF) and Ad2 pb (⁴⁷¹ VHKSFS)	
NGSYPL		Penton and penton base (fl-fiber eluted)	FN-receptor α -subunit site 5 (NGYPDL) and fiber tail (¹² NPVYPY)	

^aSequence sources were the Swiss Prot and NBRF databases, using the FASTA program for sequence homology analysis.

^bBinding significantly decreased for fiber N-terminal deletion mutants.

^cBinding significantly decreased for RGD mutants of penton base.

^dAbbreviations used are: FN, fibronectin; Ad2 E1B-55K TAG, early 1B-encoded protein of 55 kDa, identified as the T antigen (RNCCYIS); C5a, human complement C5a anaphylatoxin (KKCCYD); PSCP, human pulmonary surfactant-associated protein (TCCYIM); MAPA, rat microtubule-associated protein 1A (KPCCYIF); LIMP2, rat lysosome membrane protein II (RCCFYT).

aimed at identifying the recognition sequences involved in binding of the virion structural proteins to the cell surface receptors. We used phage-displayed peptide ligands to probe the adenovirus outer capsid reactive domains. Peptide ligands were isolated by biopanning on different affinity binding supports coated with the vertex components of the Ad2 virion. The complete penton capsomer (base + fiber) was isolated from Ad2-infected HeLa cells, whereas its constitutive domains, penton base, full-length fiber, fiber shaft and fiber knob, were obtained as recombinant proteins expressed in baculovirus-infected cells (Novelli and Boulangier, 1991b; Karayan *et al.*, 1994; Louis *et al.*, 1994). Penton base substitution mutants in the conserved RGD motif, PBR340E and PBR340G, and fiber N-terminal deletion mutants were also used in semi-quantitative assays. The search for homology of the isolated phagotope motifs to other reported protein sequences in protein databanks allowed us to arbitrarily sort the hexapeptide sequences into three different classes of proteins. The results are summarized in Table VI.

The first class (i) corresponded to peptide motifs present in Ad2 capsid components. For example, a sequence consensus to AQQHYA, which was found to bind to the penton base and fiber (Tables II and III), is found in protein IIIa, as GQEDYT, and in the precursor (PrVIII) to capsid protein pVIII (Anderson, 1990), as GAAQDYST. Likewise, NDMISR, which was biopanned on the fiber shaft (Table III), is present in PrVIII as HMISR. It is noteworthy that two peptide motifs complementary to the penton base and fiber shaft are both located within the N-terminal domain of PrVIII, corresponding to the proteolytic

fragment which has been postulated to be retained in mature virions after proteolytic cleavage at residues 112–113 by the virus-coded protease (Hannan *et al.*, 1983; Webster *et al.*, 1989).

Our isolation of mimotopes of protein IIIa and pVIII sequences by phage biopanning on penton capsomer domains suggested that proteins IIIa and pVIII are closely associated with the penton capsomer at the vertex of the adenovirion. Protein IIIa was first assigned to the apex of the viral icosahedron, like the penton base, but protein pVIII has generally been considered as being hexon associated, on the inside of the capsid (Everitt *et al.*, 1975; Pettersson, 1984). More recently a topographical model, based on image reconstruction, has located protein IIIa at the edge joining two hexon facets (Stewart *et al.*, 1993). However, our results were consistent with the recent pathway described for adenovirus uncoating and stepwise dismantling in the endosome, in which 80% of the fiber, 80% of the protein IIIa and 70% of the protein pVIII content were released from the virion within 10 min after endocytosis (Greber *et al.*, 1993). The concurrent dissociation of these three protein components strongly suggests that they are functionally (if not topographically) linked and that the fiber, protein IIIa and protein pVIII are integral parts of the viral protein complex involved in the early stages of virus–cell receptor recognition. In addition, it could not be excluded that PrVIII and protein IIIa may also interact with the penton base and fiber at certain stages of virion morphogenesis, possibly acting as scaffolding or stabilization proteins.

Interacting domains between the fiber and penton base

were also investigated, using (i) specific displacement of phages bound to wild-type full-length fiber ligate by competition with soluble penton base ligand, (ii) adsorption assays of phages on fiber wild-type and N-terminal deletion mutants and (iii) phage competition with penton assembly *in vitro*. The results obtained, along with data from penton capsomer biopanning using mAb 20G1 pre-coated affinity supports (Figure 1), suggest that the RLSNLLG motif within residues 254–260 in the penton base sequence was specific for the N-terminal 17 residues of the fiber (Table III) as previously hypothesized (Caillet-Boudin, 1989). Ligand elution from the penton base using soluble fiber yielded the highly represented NGSYPL phagotope, which, unlike GLSIWN and GQQDYA, was unique to biopanned penton base (Tables II and III). A NGSYPL-related motif (FNPVYP, residues 11–16 in Ad2 and Ad5 fibers) is highly conserved in the N-terminal tail of fibers from various serotypes (Kidd *et al.*, 1993). Although FNPVYP, which represents a true linear sequence in the fiber tail, showed a slightly higher affinity for its penton base ligand than NGSYPL (Table V), both FNPVYP and NGSYPL competed with the same efficiency with the fiber for assembly with the penton base *in vitro* (Figure 3). This strongly suggests that residues ¹¹FNPVYP¹⁶ in the tail fiber, deleted from the penton assembly-defective fiber mutants FiAT17 and FiAT61 (Karayan *et al.*, 1994), constitute a major fiber interacting site with the penton base.

The second class of phagotopes (ii) biopanned on the penton and fiber domains showed significant homology with motifs found in cellular proteins involved in host cell functions, such as receptor–ligand binding, adhesion or structural elements (Table VI). The most frequently encountered phagotopes in the eluates from both the penton base and fiber, GLSIWN and its related sequences (e.g. TRVSWN), are the consensus for a conserved motif present in the β -strand B of type III modules of human fibronectin (Bork and Doolittle, 1992; Main *et al.*, 1992; Dickinson *et al.*, 1994). A homogeneous group isolated by biopanning on the penton base, RWYRPI, is homologous to the YRGRPI motif of the fibronectin receptor α -subunit, located near to the calcium binding site (Ruoslahti, 1988). Other less represented penton base- and fiber-adsorbed phagotopes are found as homologous motifs in ankyrins (MNTLRY), cadherins (RLYDR), ICAM-1 (PQLVPS) and the LDL receptor (AHPF). For the fiber knob, adhesion or receptor motifs were also recognized, such as PRARI in fibronectin type III module 14 and PRARA in rat erythrocyte band 3 protein, which could explain the hemagglutination properties of adenoviruses and of their fibers (Rosen, 1960; Norrby, 1969). Another fiber knob binding phagotope, KLGHPN, was consensus for the KLGHPN peptide of the highly conserved cytoplasmic tyrosine kinase domain of human cell receptor tyrosine kinases (O'Bryan *et al.*, 1991; Partanen *et al.*, 1992).

In competition assays using radiolabeled Ad2 none of the phage virions carrying the consensus motifs LPRARA, GLSIWN and MTSDDL, individually or as a mixture, showed any significant reduction in Ad2 cell attachment, as a <25% decrease was observed, even at high phage input. This low competition efficiency suggested that multiple receptor sequences are involved in Ad2 cell binding and could not be competed out efficiently by one

individual or a small collection of phages. However, cell entry and nuclear delivery of Ad2 was significantly reduced (2- to 4-fold) at high phage multiplicity (Figure 4). The number of phage virions required to obtain such a detectable inhibiting effect on Ad2 entry (2000-fold the concentration of cell receptors) was consistent with the relatively low affinity of each individual phage for its viral ligand, compared with the affinity of the whole Ad2 virion for its cell receptor, which was found to be two to three orders of magnitude higher (Table V).

Taken together, our results suggest that the Ad2 penton capsomer and its two constitutive domains, the penton base and fiber, recognize multiple peptide sequences present on the cell surface, rather than one single defined receptor protein. This would explain the complexity of the Ad2 receptor sites and of the Ad2 fiber cell membrane ligands previously observed (Lonberg-Holm *et al.*, 1976; Hennache and Boulanger, 1977; Boulanger and Philipson, 1981; Defer *et al.*, 1990; Belin and Boulanger, 1993). Thus it is conceivable that adenovirus, as suggested for other viruses (reviewed in Haywood, 1994), uses a variety of binding motifs present as constitutive units of several cell membrane proteins with receptor functions. Among these Ad binding motifs the most frequently used could be: (i) the highly conserved tryptophan-containing B-element of type III modules of human fibronectin (SLLVSWQ or TMRVTW), which bound to both the fiber and penton base; (ii) a NGSYPL-like motif, consensus for the fibronectin receptor α -subunit calcium binding site 5 (NGYPDL; Ruoslahti, 1988), which was specific for the penton base.

Adsorption assay and titration of phages biopanned on penton base wild-type and RGD mutants suggested that the cellular peptide sequences complementary to the RGD peptide in the penton base belonged to the class of MTSDDL and SMDADD motifs (Tables II and IV). These two motifs are the consensus for a region of human integrin β subunits, DYPVDIYYLMDLSYSMKDDLWS-IQN, isolated by RGD cross-linking (D'Souza *et al.*, 1988, 1994). Since this region is highly conserved among all integrin β subunits, the different functions recently reported for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in Ad2 infection (Wickham *et al.*, 1994) would imply other interactions with non-RGD-containing domain(s) of the penton base or/and with other capsid proteins.

The third class (iii) consisted of peptide sequences common to the two previous classes and shared by virus and cell components. This was the case for NGSYPL, THSKSF and KKCCYI-KCCFYV (Table VI). Our finding of a sequence homology between a penton base binding motif in the fiber tail (FNPVYP-NGSYPL) and linear peptides found in the fibronectin receptor α -subunit (NGYPDL) and in the LDL receptor (endocytosis signal FDNPVY) suggests that competition for similar binding sites and an exchange of protein ligands between host cell and virion components could occur during virus attachment, endocytosis and uncoating. The primary event in Ad uncoating is generally thought to be labilization of the Ad capsid, which results from fiber attachment to its cell surface receptor. Indeed, Ad2 penton capsomer has been found to dissociate into fiber and penton base upon reaction with antibody directed against the fiber (Boudin and Boulanger, 1981, 1982). Thus, following release of

the fiber tail, the penton base unoccupied site could bind to an equivalent motif in the fibronectin receptor α -chain or the LDL receptor.

A similar mechanism of cell-virus ligand exchange could also take place at the stage of virus assembly and release from the host cell at the end of its life cycle. The finding that the THSKSF motif, eluted from the fiber knob, exists in the penton base (as VHKSFS) and has an equivalent in some cell receptors (RESKSF in the human C5a receptor) is probably not coincidental. It is reminiscent of a previous model for intranuclear crystals of adenovirus particles in which the distance between the virions and their symmetry within the crystal unit cell imposed interactions between the fiber knob and penton base (Boulanger *et al.*, 1974). The fiber knob binding motif, VHKSFS, in the penton base might act within the cell to prevent the fiber attaching prematurely to a cell component(s). Likewise, the KKCCYI-KCCFYV motif, specific for the fiber knob-shaft junction, is present in the Ad early 1B-encoded 55 kDa protein known as the T antigen, which has a function in cell transformation and a role in the expression of viral late structural proteins (Yew *et al.*, 1990). It is also found in lysosome membrane protein II and rat microtubule-associated protein 1A, as well as in human extracellular proteins involved in host defense, e.g. complement C5a anaphylatoxin and pulmonary surfactant-associated protein (Table VI). The host-virus ligand exchange mechanism might therefore offer a clue to elucidate the general problem of the virus assembly-disassembly paradox recently evoked by Helenius (1992).

The results of our biopanning shed light on some earlier observations on adenovirus physiopathology. Ten years ago it was reported that adenovirus induced the release of gold-labeled EGF into the cytosol of KB cells during receptor-mediated endocytosis (FitzGerald *et al.*, 1983) and this effect was attributed to penton base (Seth *et al.*, 1984, 1985, 1986; Seth, 1994). The finding of a motif with high homology to the EGF receptor in our penton base biopanning (VEPATP-VEPLTP; Tables II and VI) raises the hypothesis that the penton base, after release from the virion after endocytosis, competes with EGF to occupy the same receptor, resulting in EGF escape into the cytosol. This offers a clue into the understanding of the molecular mechanisms of vesicular escape from endosomes, which constitutes a key parameter for efficient gene delivery using adenoviral vectors.

Materials and methods

Cells, viruses and Ad2 capsid proteins

HeLa cells were grown as monolayers and infection with wild-type Ad2 was carried out as previously described (Defer *et al.*, 1990). The Ad2 penton was isolated from the cellular pool of soluble antigens and purified by conventional techniques (Boulanger and Puvion, 1973). Insect cells (Sf9) were also grown as monolayers and baculovirus (AcMNPV) recombination protocols, infection and expression of recombinant proteins have been described in detail in previous studies. Penton base (PbFL571), full-length fiber (FiFL582), fiber shaft (FiDT61-410) and fiber knob (FiAT388) were produced as recombinant Ad2 capsid proteins in baculovirus-infected Sf9 cells (Luckow and Summers, 1989) and isolated as previously described (Boudin *et al.*, 1979; Novelli and Boulanger, 1991b; Karayan *et al.*, 1994). The recombinant AcMNPV clone expressing the Ad2 fiber knob was obtained from J.Chroboczek (Louis *et al.*, 1994). To be consistent with our previous nomenclature (Novelli and Boulanger, 1991b), the fiber knob was referred to as

FiAT388, since it was deleted of the N-terminal 387 residues. Baculovirus recombinants expressing penton base mutants carrying an Arg→Glu substitution at position 340 in the RGD motif (mutant PbR340E) or an Arg→Gly substitution at the same position (mutant PbR340G) were generated by site-directed mutagenesis, using the single-stranded DNA method of Kunkel *et al.* (1987) with R408 as the helper phage (Russell *et al.*, 1986). They will be described in detail elsewhere (manuscript in preparation).

Antibodies

Polyclonal anti-penton base serum (laboratory made) was raised in rabbit by injection of purified recombinant penton base in its pentameric form (9S fraction; Karayan *et al.*, 1994). Mouse anti-penton base mAb 20G1 was isolated in our laboratory and partially characterized (Karayan *et al.*, 1994). Mouse mAb 4D2.5, directed against both the Ad2 and Ad5 fiber N-terminal regions (Hong and Engler, 1991), was a kind gift from J.Engler.

Phage hexapeptide library and affinity biopanning techniques

The filamentous phage hexapeptide library was kindly provided by Prof. George Smith (Scott and Smith, 1990). Affinity biopanning of phages binding to a ligate of choice was carried out following published protocols (Parmley and Smith, 1988; Smith and Scott, 1993), with some modifications. The modification we introduced was in the preparation of the affinity supports used for biopanning and in the elution protocols.

Affinity supports with randomly adsorbed ligates. When the ligate to be biopanned was a mAb or an Ad2 capsid protein in a non-specific orientation they were coated directly onto microtiter plate wells (Nunc-ImmunoModule MaxiSorp F8) at a concentration of 1 μ g/well in 0.1 M sodium carbonate buffer, pH 9.6, overnight at 4°C.

Affinity supports with specific orientation of the immobilized ligate. When specific orientation of the penton ligate was desired the viral protein was allowed to adsorb in excess onto a surface which had been pre-coated with anti-penton base antibody. This was achieved by pre-coating anti-penton base rabbit polyclonal antibody or mAb (0.5–1 μ g/well) onto microtiter plate wells, followed by adsorption of Ad2 penton (to saturation). The wells were then washed three times with TBS containing 0.5% Tween-20 (TBST) and blocked by incubating for 90 min at room temperature with 3% bovine serum albumin in TBS. Subsequently, 50 μ l (corresponding to 10^{10} phage particles) of the phage library were added and allowed to adsorb for 2 h at room temperature. The wells were then rinsed 10 times with TBST to remove excess and unbound phages.

Low pH elution. Total elution of phages bound to the affinity surface was performed using 0.1 N HCl buffered to pH 2.2 with glycine and containing 1 mg/ml bovine serum albumin (Parmley and Smith, 1988). Elution was carried out for 10 min at room temperature.

Ligand elution. Selective elution was performed using a partner protein acting as a competing ligand (Smith and Scott, 1993). This was referred to as ligand elution, e.g. Ad2 penton base protein was used to ligand elute phages adsorbed on Ad2 fiber and vice versa. The ligands used for elution (baculovirus-expressed recombinant penton base and fiber) were isolated from serum-free culture media in which they are secreted in a soluble, native form (Novelli *et al.*, 1991b; Karayan *et al.*, 1994). Elution of bound phages was carried out for 1 h at 37°C and the eluted phages were immediately amplified in K91Kan bacteria and partially purified by polyethylene glycol precipitation. Three rounds of biopanning were carried out before the affinity-purified phages were isolated on LB agar plates supplemented with 40 μ g/ml tetracycline (Tet) and 100 μ g/ml kanamycin (Kan).

Phage adsorption assay

This assay, also known as 'micropanning' has been described by Parmley and Smith (1988) to screen for tight binding phages after biopanning. We have used this assay to quantitate the binding capacity of selected phages on various ligands. Phages were adsorbed at a concentration of 1×10^5 virions/ml onto 96-well microtiter plates coated with the ligate of interest, the penton base or fiber, wild-type or mutant. After 30 min incubation at room temperature unadsorbed phages were removed and the wells washed 10 times with TBS. Elution was carried out either with 20 μ l acid elution buffer (10 min, room temperature) or using a competing ligand (30 min, 37°C) and eluates were transferred to corresponding wells of new 96-well plates containing 2 μ l 1.25 M Tris-HCl, pH 9.2. Then 5 μ l concentrated suspension of K91Kan cells were

added to each well and the plates incubated for 15 min at 37°C. Subsequently 20 µl LB was added to each well and the plates further incubated for another 15 min. Aliquots (20 µl) from each well were then spotted onto LB plates containing Tet and Kan. Colonies were counted from each spot after 16 h growth at 37°C.

DNA sequence analysis

The sequences of the hexapeptide-harboring recombinant phage pIII proteins were determined after isolation of single-stranded DNA from fUSE5 phages and sequencing performed using the dideoxynucleotide chain termination method (Sanger *et al.*, 1987) and the oligonucleotide 5'-TGAATTTTCTGTATGAGG-3' as primer. The Sequenase kit version 2.0 (Amersham) was used for sequencing, according to the supplier's recommendations. Unless homogeneous sequences were found in the first 10 phage clones, 24–30 individual clones were usually sequenced.

Search for sequence homology

Hexapeptides isolated were compared with the 33 000 entries of the SwissProt protein database, using the FASTA 1.6 program (Pearson and Lipman, 1988) and the BISANCE system (Dessen *et al.*, 1990).

Binding assays

For determination of relative affinities between phages and ligands aliquots of phage virions were immobilized on a solid phase by adsorption onto microtiter plates at 5×10^{10} virions/well for 16 h at 4°C. After rinsing with TBS buffer serial dilutions of isotopically labeled recombinant fiber or the penton base in TBS (100 µl aliquot/well) were incubated with phages for 1 h at 25°C. The wells were washed twice with TBST and all the washing fractions were pooled. Phage-bound ligand was recovered with acidic elution buffer, as described above. Trichloroacetic acid (TCA)-precipitable radioactivity of phage-bound and unbound fractions was collected on GF/C filters and determined by counting in a liquid scintillation spectrometer (Beckman scintillation counter LS-6500). The values of the ratio of bound to unbound counts were plotted versus bound counts and the apparent association constants were derived from the slope of the plots (Scatchard, 1949). The recombinant wild-type penton base (PbFL571) and fiber (FiFL582) used as ligands were labeled with both [³⁵S]methionine and [³⁵S]cysteine. Trans³⁵S-label (ICN, Irvine, CA; 40 TBq/mmol) was added to baculovirus-infected Sf9 cell cultures, maintained in methionine-free medium, at 740 kBq/ml for 24 h at 16 h after infection (Karayan *et al.*, 1994). The specific radioactivity ranged between 15 000 and 30 000 c.p.m./µg recombinant penton base and fiber.

In vitro assembly of the penton base and fiber was carried out by incubating FiFL582 fiber with PbFL571 penton base in stoichiometric amounts (0.2 µg each recombinant protein in 10 µl TBS buffer) at 25°C for 4 h in the presence of increasing concentrations of phage virions in 10 µl aliquots of TBS, ranging from 10^5 to 10^{12} virions/sample. At the end of the incubation period 15 µl 40% glycerol solution in TBS was added and the efficiency of penton assembly *in vitro* was estimated by gel mobility shift assay using electrophoresis in non-denaturing 6% polyacrylamide gels (Karayan *et al.*, 1994). Free fiber migrates as a diffuse band, whereas penton base-associated fiber is retarded and migrates as a discrete band of penton, reacting with both fiber and penton base antibodies in immunoblots. After electric transfer of proteins the nitrocellulose membranes (Hybond ECL, Amersham) were successively reacted with rabbit anti-fiber antibody (1:5000 dilution; laboratory made), horseradish peroxidase-labeled anti-rabbit IgG conjugate (1:2000 dilution; Amersham) and chemiluminescent substrate (Boehringer Mannheim) and exposed to radiographic films (Hyperfilm-βmax, Amersham). The amount of fiber in the penton band was quantitated by densitometric scanning of luminograms at 610 nm, using an automatic densitometer system (REP-EDC; Helena Laboratories, Beaumont, TX).

Adenovirus particle attachment to cell plasma membrane receptors was carried out at 4°C for 2 h, using [¹⁴C]valine-labeled Ad2 virions and HeLa cell monolayers grown in 24-well plates (5×10^5 cells/well), as previously described (Belin and Boulanger, 1993). Virus entry was analyzed by incubating [¹⁴C]valine-labeled Ad2 virions with HeLa cell monolayer samples at 37°C for 1 h. Aliquots of 1×10^5 virions/cell were used, representing a 10-fold excess over the estimated maximum number of cell membrane receptors (5000–10 000 per cell; Persson *et al.*, 1985; Defer *et al.*, 1990). The specific radioactivity of Ad2 virions used was 0.9×10^5 – 1.0×10^5 c.p.m./ 10^{12} particles and the virus inoculum usually contained 2×10^{12} virions/ml. In competition experiments Ad2 samples were pre-incubated at 25°C for 1 h with 10^9 , 10^{10} , 10^{11} or 10^{13} phage virions, corresponding to 1-, 2-, 20- and 2000-fold the theoretical number of cell receptors respectively. In some cell adsorption assays soluble phage pIII proteins were used in lieu of phage virions. Suspensions of

phage virions (10^{14} /ml) were incubated at 56°C for 1 min in TBS containing 0.5% sodium deoxycholate (DOC) and the samples clarified at 8000 g for 10 min. The tip protein pIII was quantitatively recovered in the supernatant, along with the pVIII major capsid protein (unpublished data). Aliquots of DOC-solubilized phage pIII protein, corresponding to 10^{10} , 10^{11} and 10^{13} phage virions of the starting material respectively, were mixed with radiolabeled Ad2 and the mixture overlaid onto HeLa cell monolayers. The quantity of membrane-adsorbed Ad2 at 4°C and the quantity of total virus uptake at 37°C were both determined by counting the TCA-precipitable radioactivity of solubilized whole cell pellets. The amount of endosomal and nucleus-associated counts were determined after cell fractionation, as previously described (Defer *et al.*, 1990). Phage concentration was determined by a spectrophotometric assay, assuming that 30 OD_{269 nm} U/ml is equivalent to 2×10^{14} phage virions (Smith and Scott, 1993).

Acknowledgements

We are grateful to Jacqueline Gerfaux for penton base mutant plasmids, to Jadwiga Chroboczek for her Ad2 fiber knob recombinant baculovirus and Marie-Thérèse Belin for her Ad2 stock. We also thank Martine Bardy for her help with the cell cultures, Bernard Gay for photography, Christiane Senglat, Véronique Parra and Raymond Baudoin for technical support and Liliane Cournud for secretarial aid. This work was supported in part by the Centre National de la Recherche Scientifique (URA 1487), the Association Française de Lutte contre la Mucoviscidose (AFLM–Artemis Program), the Fondation pour la Recherche Médicale (FRM) and Transgene SA (Strasbourg). S.S.H. was the recipient of a fellowship from the FRM (1992–1993) and AFLM (1994–1995).

References

- Anderson, C.W. (1990) The proteinase polypeptide of adenovirus serotype 2 virions. *Virology*, **177**, 259–272.
- Bai, M., Harfe, B. and Freimuth, P. (1993) Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. *J. Virol.*, **67**, 5198–5205.
- Bai, M., Campisi, L. and Freimuth, P. (1994) Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. *J. Virol.*, **68**, 5925–5932.
- Belin, M.T. and Boulanger, P. (1993) Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface. *J. Gen. Virol.*, **74**, 1485–1497.
- Blond-Elguindi, S., Cwirla, S.E., Dower, W.J., Lipshutz, R.J., Sprang, S.R., Sambrook, J.F. and Gething, M.J.H. (1993) Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell*, **75**, 717–728.
- Bork, P. and Doolittle, R.F. (1992) Proposed acquisition of an animal protein domain by bacteria. *Proc. Natl Acad. Sci. USA*, **89**, 8990–8994.
- Boudin, M.L. and Boulanger, P. (1981) Antibody-triggered dissociation of adenovirus penton capsomer. *Virology*, **113**, 781–786.
- Boudin, M.L. and Boulanger, P. (1982) Assembly of adenovirus penton base and fiber. *Virology*, **116**, 589–604.
- Boudin, M.L., Moncany, M., D'Halluin, J.C. and Boulanger, P. (1979) Isolation and characterization of adenovirus type 2 vertex capsomer (penton base). *Virology*, **92**, 125–138.
- Boudin, M.L., D'Halluin, J.C., Cousin, C. and Boulanger, P. (1980) Human adenovirus type 2 protein IIIa. II. Maturation and encapsidation. *Virology*, **101**, 144–156.
- Boulanger, P. and Philipson, L. (1981) Membrane components interacting with non-enveloped viruses. In Lonberg-Holm, K. and Philipson, L. (eds), *Receptors and Recognition*, Series B, vol. 8, *Virus Receptors*, Part 2, *Animal Viruses*. Chapman and Hall, New York, NY, pp. 117–139.
- Boulanger, P. and Puvion, F. (1973) Large scale preparation of soluble adenovirus hexon, penton and fiber antigens in highly purified form. *Eur. J. Biochem.*, **39**, 37–42.
- Boulanger, P., Torpier, G. and Rimsky, A. (1974) Crystallographic study of intranuclear adenovirus type 5 crystals. *Intervirology*, **2**, 56–62.
- Caillet-Boudin, M.L. (1989) Complementary peptide sequences in partner proteins of the adenovirus capsid. *J. Mol. Biol.*, **208**, 195–198.
- Cuillet, M., Cortolezzi, B., Chroboczek, J., Langowski, J., Ruigrok, R.W.H. and Jacrot, B. (1990) Purification and characterization of wild type and ts112 mutant protein IIIa of human adenovirus 2 expressed in *Escherichia coli*. *Virology*, **175**, 222–231.

- Cuzange,A., Chroboczek,J. and Jacrot,B. (1994) The penton base of human adenovirus type 3 has the RGD motif. *Gene*, **146**, 257–259.
- Cwirla,S.E., Peters,E.A., Barrett,R.W. and Dower,W.J. (1990) Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl Acad. Sci. USA*, **87**, 6378–6382.
- Defer,C., Belin,M.T., Caillet-Boudin,M.L. and Boulanger,P. (1990) Human adenovirus–host cell interactions: a comparative study with members of subgroups B and C. *J. Virol.*, **64**, 3661–3673.
- Dessen,P., Fondrat,C., Valencien,C. and Mugnier,C. (1990) BISANCE: a French service for access to biomolecular sequence databases. *Curr. Adv. Biol. Sci.*, **6**, 355–356.
- Devaux,C., Caillet-Boudin,M.L., Jacrot,B. and Boulanger,P. (1987) Crystallization, enzymatic cleavage and the polarity of the adenovirus fibre. *Virology*, **161**, 121–128.
- Dickinson,C.D., Veerapandian,B., Dai,X.-P., Hamlin,R.C., Xuong,N.-H., Ruoslahti,E. and Ely,K.R. (1994) Crystal structure of the tenth type III cell adhesion module of human fibronectin. *J. Mol. Biol.*, **236**, 1079–1092.
- D'Souza,S.E., Ginsberg,M.H., Burke,T.A., Lam,S.C.-T. and Plow,E.F. (1988) Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science*, **242**, 91–93.
- D'Souza,S.E., Haas,T.A., Piotrowicz,R.S., Byers-Ward,V., McGrath,D.E., Soule,H.R., Ciermiewski,C., Plow,E.F. and Smith,J.W. (1994) Ligand and cation binding are dual functions of a discrete segment of the integrin $\beta 3$ subunit: cation displacement is involved in ligand binding. *Cell*, **79**, 659–667.
- Everitt,E., Lutter,L. and Philipson,L. (1975) Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirus type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. *Virology*, **67**, 197–208.
- FitzGerald,D.J.P., Padmanabhan,R., Pastan,I. and Willingham,M.C. (1983) Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell*, **32**, 607–617.
- Folgori,A., Tafi,R., Meola,A., Felici,F., Galfré,G., Cortese,R., Monaci,P. and Nicosia,A. (1994) A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera. *EMBO J.*, **13**, 2236–2243.
- Greber,U.F., Willetts,M., Webster,P. and Helenius,A. (1993) Stepwise dismantling of adenovirus 2 entry into cells. *Cell*, **75**, 477–486.
- Green,N.M., Wrigley,N.G., Russell,W.C., Martin,S.R. and MacLachlan,A.D. (1983) Evidence for a repeating cross- β sheet structure in the adenovirus fibre. *EMBO J.*, **2**, 1357–1365.
- Hannan,C., Raptis,L.H., Déry,C.V. and Weber,J. (1983) Biological and structural studies with an adenovirus type 2 temperature-sensitive mutant defective for uncoating. *Intervirology*, **19**, 213–223.
- Haywood,A.M. (1994) Virus receptors: binding, adhesion strengthening and changes in viral structure. *J. Virol.*, **68**, 1–5.
- Helenius,A. (1992) Unpacking the incoming influenza virus. *Cell*, **69**, 577–578.
- Hennache,B. and Boulanger,P. (1977) Biochemical study of KB cell receptor for adenovirus. *Biochem. J.*, **166**, 237–247.
- Henry,L., Xia,D., Wilke,M., Deisenhofer,J. and Gerard,R.D. (1994) Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *E. coli*. *J. Virol.*, **68**, 5239–5246.
- Hong,J.S. and Engler,J.A. (1991) The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal. *Virology*, **185**, 758–767.
- Karayan,L., Gay,B., Gerfaux,J. and Boulanger,P. (1994) Oligomerization of recombinant penton base of adenovirus type 2 and its assembly with fiber in baculovirus-infected cells. *Virology*, **202**, 782–796.
- Kidd,A.H., Chroboczek,J., Cusack,S. and Ruigrok,R.W. (1993) Adenovirus type 40 virions contain two distinct fibers. *Virology*, **192**, 73–84.
- Koivunen,E., Gay,D.A. and Ruoslahti,E. (1993) Selection of peptides binding to the $\alpha 5\beta 1$ integrin from a phage display library. *J. Biol. Chem.*, **268**, 20205–20210.
- Koivunen,E., Wang,B. and Ruoslahti,E. (1994) Isolation of a highly specific ligand for the $\alpha 5\beta 1$ integrin from a phage display library. *J. Cell Biol.*, **124**, 373–380.
- Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–383.
- Lonberg-Holm,K. and Philipson,L. (1969) Early events of virus–cell interaction in an adenovirus system. *J. Virol.*, **4**, 323–338.
- Lonberg-Holm,K., Crowell,R.L. and Philipson,L. (1976) Unrelated animal viruses share receptors. *Nature (Lond.)*, **259**, 679–681.
- Louis,N., Fender,P., Barge,A., Kitts,P. and Chroboczek,J. (1994) Cell-binding domain of adenovirus serotype 2 fiber. *J. Virol.*, **68**, 4104–4106.
- Luckow,V.A. and Summers,M.D. (1989) High level expression of nonfused foreign genes with *Autographa californica* Nuclear Polyhedrosis Virus expression vectors. *Virology*, **170**, 31–39.
- Main,A.L., Harvey,T.S., Baron,M., Boyd,J. and Campbell,I.D. (1992) The three-dimensional structure of the tenth type III module of fibronectin: an insight into RGD-mediated interactions. *Cell*, **71**, 671–678.
- Mathias,P., Wickham,T., Moore,M. and Nemerow,G. (1994) Multiple adenovirus serotypes use α_V integrins for infection. *J. Virol.*, **68**, 6811–6814.
- Nemerow,G.R., Cheresh,D.A. and Wickham,T.J. (1994) Adenovirus entry into host cells: a role for α_V integrins. *Trends Cell Biol.*, **4**, 52–55.
- Nermut,M.V. (1984) The architecture of adenoviruses. In Ginsberg,H.S. (ed.), *The Adenoviruses*. Plenum, New York, NY, pp 5–34.
- Neuman,R., Chroboczek,J. and Jacrot,B. (1988) Determination of the nucleotide sequence for the penton-base gene of human adenovirus type 5. *Gene*, **69**, 153–157.
- Norrby,E. (1969) The structural and functional diversity of adenovirus capsid components. *J. Gen. Virol.*, **5**, 221–236.
- Novelli,A. and Boulanger,P. (1991a) Assembly of adenovirus type 2 fiber synthesized in cell-free translation system. *J. Biol. Chem.*, **266**, 9299–9303.
- Novelli,A. and Boulanger,P. (1991b) Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. *Virology*, **185**, 365–376.
- O'Bryan,J.P., Frye,R.A., Cogswell,P.C., Neubauer,A., Kitch,B., Prokop,C., Espinoza,R., Le Beau,M.M., Earp,H.S. and Liu,E.T. (1991) *axl*, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Mol. Cell. Biol.*, **11**, 5016–5031.
- Oldenburg,K.R., Loganathan,D., Goldstein,I., Schultz,P.G. and Gallop,M.A. (1992) Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc. Natl Acad. Sci. USA*, **89**, 5393–5397.
- Parnley,S.F. and Smith,G.P. (1988) Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene*, **73**, 305–318.
- Partanen,J., Armstrong,E., Makela,T.P., Korhonen,J., Sandberg,M., Renkonen,R., Knuutila,K. and Alitalo,K. (1992) A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. *Mol. Cell. Biol.*, **12**, 1698–1707.
- Pearson,W.R. and Lipman,D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.
- Persson,R., Svensson,U. and Everitt,E. (1985) Virus receptor interaction in the adenovirus system: characterization of the positive cooperative binding of virions on HeLa cells. *J. Virol.*, **54**, 92–97.
- Pettersson,U. (1984) Structural and non-structural adenovirus proteins. In Ginsberg,H.S. (ed.), *The Adenoviruses*. Plenum, New York, NY, pp 205–270.
- Roberts,R.J., Akusjärvi,G., Aleström,P., Gelinis,R.E., Gingeras,T.R., Sciaky,D. and Pettersson,U. (1986) A consensus sequence for the adenovirus 2 genome. In Doerfler,W. (ed.), *Adenovirus DNA: The Viral Genome and its Expression*. Martinus Nijhoff, Boston, MA, pp. 1–51.
- Rosen,L. (1960) A hemagglutination-inhibition technique for typing adenoviruses. *Am. J. Hyg.*, **71**, 120–128.
- Ruigrok,R.W.H., Barge,A., Albiges-Rizo,C. and Dayan,S. (1990) Structure of adenovirus fibre. II. Morphology of single fibres. *J. Mol. Biol.*, **215**, 589–596.
- Ruoslahti,E. (1988) Fibronectin and its receptors. *Annu. Rev. Biochem.*, **57**, 375–413.
- Russel,M., Kidd,S. and Kelley,M.R. (1986) An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene*, **45**, 333–338.
- Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
- Scatchard,G. (1949) The attraction of proteins for small molecules and ions. *Annals NY Acad. Sci.*, **51**, 660–672.
- Scott,J.K. and Smith,G.P. (1990) Searching for peptide ligands with an epitope library. *Science*, **249**, 386–390.
- Scott,J.K., Loganathan,D., Easley,R.B., Gong,X. and Goldstein,I.J. (1992) A family of concanavalin A-binding peptides from a hexapeptide epitope library. *Proc. Natl Acad. Sci. USA*, **89**, 5398–5402.
- Seth,P. (1994) Adenovirus-dependent release of choline from plasma

- membrane vesicles at an acidic pH is mediated by the penton base protein. *J. Virol.*, **68**, 1204–1206.
- Seth,P., FitzGerald,D., Ginsberg,H.S., Willingham,M. and Pastan,I. (1984) Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor. *Mol. Cell. Biol.*, **4**, 1528–1533.
- Seth,P., Pastan,I. and Willingham,M. (1985) Adenovirus-dependent increase in cell membrane permeability. *J. Biol. Chem.*, **260**, 9598–9602.
- Seth,P., FitzGerald,D., Willingham,M. and Pastan,I. (1986) Pathway of adenovirus entry into cells. In Crowell,R.L. and Lonberg-Holm,K. (eds), *Virus Attachment and Entry into Cells*. American Society for Microbiology, Washington, DC, pp. 191–195.
- Signäs,C. Akusjärvi,G. and Pettersson,I. (1985) Adenovirus 3 fiber polypeptide gene: implication for the structure of the fiber protein. *J. Virol.*, **53**, 672–678.
- Smith,G.P. and Scott,J.K. (1993) Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol.*, **217**, 228–257.
- Sprengel,J., Schmitz,B., Heuss-Neitzel,D., Zock,C. and Doerfler,W. (1994) Nucleotide sequence of human adenovirus type 12 DNA: comparative functional analysis. *J. Virol.*, **68**, 379–389.
- Stephen,C.W. and Lane,D.P. (1992) Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. *J. Mol. Biol.*, **225**, 577–583.
- Stewart,P.L., Burnett,R.M., Cyrklaff,M. and Fuller,S.D. (1991) Image reconstruction reveals the complex molecular organization of adenovirus. *Cell*, **67**, 145–154.
- Stewart,P.L., Fuller,S.D. and Burnett,R.M. (1993) Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.*, **12**, 2589–2599.
- Stouten,P.F.W., Sander,C., Ruigrok,R.W.H. and Cusack,S. (1992) A new triple-helical model for the shaft of the adenovirus fibre. *J. Mol. Biol.*, **226**, 1073–1084.
- Svensson,U., Persson,R. and Everitt,E. (1981) Virus–receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. *J. Virol.*, **38**, 70–81.
- Valentine,R.C. and Pereira,H.G. (1965) Antigens and the structure of the adenovirus. *J. Mol. Biol.*, **13**, 13–20.
- van Oostrum,J. and Burnett,R.M. (1985) Molecular composition of the adenovirus type 2 virion. *J. Virol.*, **56**, 439–448.
- van Oostrum,J., Smith,P.R., Mohraz,M. and Burnett,R.M. (1987) Molecular composition of the structure of the adenovirus capsid. III. Hexon packing determined from electron micrographs of capsid fragments. *J. Mol. Biol.*, **198**, 73–89.
- Varga,M., Weibull,C. and Everitt,E. (1991) Infectious entry pathway of adenovirus type 2. *J. Virol.*, **65**, 6061–6070.
- Weber,J., Talbot,B.G. and Delorme,L. (1989) The orientation of the adenovirus fiber and its anchor domain identified through molecular mimicry. *Virology*, **168**, 180–182.
- Webster,A., Russell,S., Talbot,P., Russell,W.C. and Kemp,G.D. (1989) Characterization of the adenovirus proteinase: substrate specificity. *J. Gen. Virol.*, **70**, 3225–3224.
- White,J.M. (1993) Integrins as virus receptors. *Curr. Biol.*, **3**, 596–599.
- Wickham,T.J., Mathias,P., Cheresh,D.A. and 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. and Nemerow,G.R. (1994) Integrin $\alpha_v\beta_5$ selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.*, **127**, 257–264.
- Wilcox,W.C. and Ginsberg,H.S. (1963) The structure of type 5 adenovirus. I. Antigenic relationship of virus structural proteins to virus specific soluble antigens from infected cells. *J. Exp. Med.*, **118**, 295–306.
- Xia,D., Henry,L.J., Gerard,R.D. and Deisenhofer,J. (1994) Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. *Curr. Biol. Struct.*, **2**, 1259–1270.
- Yayon,A., Aviezer,D., Safran,M., Gross,J.L., Heldman,Y., Cabilly,S., Givol,D. and Katchalski-Katzir,E. (1993) Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage-epitope library. *Proc. Natl Acad. Sci. USA*, **90**, 10643–10647.
- Yew,P.R., Kao,C.C. and Berk,A.J. (1990) Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. *Virology*, **179**, 795–805.

Received on April 5, 1995; revised on June 29, 1995