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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

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The adenovirus penton base protein has a cell rounding activity and may lyse endosomes during virus entry into the cytoplasm. We found that penton base that was expressed in *Escherichia coli* also caused cell rounding and that cells adhered to polystyrene wells that were coated with the protein. Mutant analysis showed that both properties required an Arg-Gly-Asp (RGD) sequence at residues 340 to 342 of penton base. In flat adherent cells, virus mutants with amino acid substitutions in the RGD sequence were delayed in virus reproduction and in the onset of viral DNA synthesis. In nonadherent or poorly spread cells, the kinetics of mutant virus reproduction were similar to those of wild-type adenovirus type 2. Expression of the mutant phenotype exclusively in the flat cells that we tested supports a model in which penton base interacts with an RGD-directed cell adhesion molecule during adenovirus uptake or uncoating.

Adenoviruses are nonenveloped particles that must cross a cell membrane system to initiate infection, but the mechanism of this process has not been determined. Only one virion protein, the fiber, has been shown to interact directly with the cell membrane. Fibers are elongated structures that project from the virion surface (21). One trimer of 62-kDa fiber subunits occurs at each of the 12 vertices of the capsid, where it is noncovalently associated with the pentameric vertex capsomer, the penton base (21, 42). The distal end of the fiber consists of a globular domain which presumably directs the attachment of virus to a specific receptor in the plasma membrane (23–25). Adsorption of virions occurs through this interaction and can be blocked by saturating receptors with purified fiber subunits (25). Soon after adsorption, the virus-receptor complex diffuses into coated pits and is internalized by endocytosis (7, 10).

A possible interaction between penton base and the cell membrane is suggested by earlier observations that adherent cells round up and detach when exposed to purified penton base-fiber complexes (9, 22, 28). Only a fraction of the pentons synthesized during infection assemble into virions, and the cell rounding activity first was observed in lysates of infected cells that were used as crude virus stocks. Subsequently the activity was found associated with penton base subunits that accumulated in cells infected with fiber temperature-sensitive mutants (2). Cellular DNA and protein synthesis are not significantly affected by exposure of cells to penton base, indicating that penton base is not cytotoxic (13). The mechanism and physiologic role of this activity have not been determined.

Data consistent with the model that adenovirus enters the cytoplasm from disrupted endosomes have been reported. Electron micrographs show virus in coated pits, in intracellular vesicles, and free in the cytoplasm adjacent to membrane fragments (7, 10, 39, 40). Infection appears to cause leakage from endosomes of molecules that have been coendocytosed with virus and has been demonstrated with fluoresceinated dextran (8), toxin-epidermal growth factor conjugates (10), or DNA (46). This property is not conserved in all adenoviruses; it was observed in cells infected with adenoviruses in subgroup C (serotypes 2 and 5) but not in cells infected with subgroup B viruses (serotypes 4 and 7) (8). Release of reporter molecules into the cytoplasm was blocked by treatment of cells with chloroquine (35) or by antibodies against penton base (33), and an acid-induced hydrophobic conformation subsequently was demonstrated in penton base (39, 45). These results support the hypothesis that the endosomal membrane is disrupted by hydrophobic surfaces that become exposed on the viral penton base subunits at an acid pH (34). This unique entry pathway has focused attention recently on the use of adenovirus as a vector for gene therapy (6, 46).

Here we report evidence that an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 (Ad2) penton base is a ligand for a cell adhesion receptor. This ligand occurs in a hypervariable region of penton base and is conserved in some but not all adenovirus serotypes. Mutants of Ad2 with amino acid substitutions in the RGD sequence were constructed. Reproduction and DNA synthesis of the mutants proceeded with normal kinetics in cell lines that fail to adhere or spread on plastic dishes but were delayed by 5 to 10 h in flat, tightly adherent cells. Coinfection with wild-type Ad2 failed to complement the delay in mutant DNA synthesis. Our data suggest that an interaction between the penton base in virions and a cell adhesion molecule may accelerate the entry or uncoating of virus in flat cells.

MATERIALS AND METHODS

Cells and virus. The cell lines used in these studies were HeLa S3 suspension cells, two subclones of adherent HeLa cells, DK and JW, derived by D. Klessig (Rutgers University) and J. Williams (Carnegie-Mellon University), 293 cells (14), and A549 cells (ATCC CCL 185). HeLa S3 cells were grown in suspension culture in F13 medium supplemented with 5% calf serum. All other cells were grown on plastic tissue culture dishes in Dulbecco's minimum essential me-

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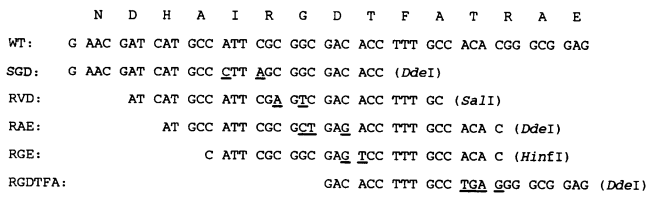

FIG. 1. Mutagenic oligonucleotides. Mismatches are underlined, and novel restriction sites are indicated in parentheses. WT, wild type.

dium (DME) supplemented with 10% calf serum in an atmosphere of 5% CO_2. At 16 to 20 h before infection, adherent cells were trypsinized and replated at 50% confluence. HeLa S3 cells were subcultured three times per week and were used when the stock culture concentration was between 3.5×10^5 and 5×10^5 cells per ml. Infected cells were maintained in medium containing 2% calf serum. Ad2 was obtained from C. Anderson (Brookhaven National Laboratory). Virus titers were determined on A549 cell monolayers in 24-well plates (Costar) by indirect immunoassay 40 h after infection, using rabbit anti-Ad2 hexon serum and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Cappel). Viral DNA was isolated from infected cells that were lysed in buffer containing 0.1% sodium dodecyl sulfate (SDS) by a modified Hirt extraction procedure as described elsewhere (41). DNA samples were dissolved in 50 μl of Tris-EDTA buffer. Equal volumes (3 μl) of each sample were digested with restriction enzymes and electrophoresed in agarose gels to study viral DNA synthesis. Viral DNA-terminal protein complexes were isolated from CsCl-purified Ad2 virions. Virus was disrupted by mixing with an equal volume of saturated guanidine-HCl and was chromatographed on Sepharose 4B-CL equilibrated in 4 M guanidine-HCl–10 mM Tris-HCl (pH 8)–0.1 mM EDTA. DNA-terminal protein complexes eluted in the void volume and were dialyzed against Tris-EDTA buffer and stored at −70°C. Penton and fiber subunits were purified from infected HeLa S3 cells according to the procedure of Boulanger and Puvion (3).

Construction of virus mutants. The Ad2 *Hin*dIII-*Bam*HI restriction fragment from nucleotides 15032 to 15403 was cloned in M13mp18 and was mutagenized according to the protocol of Kunkel (18) with oligonucleotides (Fig. 1) that simultaneously encoded amino acid substitutions in the RGD sequence and introduced novel restriction sites. The DNA sequence of the entire *Hin*dIII-*Bam*HI fragment of each mutant was determined by using vector-specific primers and a Sequenase II chain termination kit from U.S. Biochemical. The mutations were transferred into successively larger subclones of Ad2 DNA (the *Sma*I-*Sal*I fragment from nucleotides 14456 to 16744 and the *Xba*I-*Sal*I fragment from nucleotides 10579 to 16744, both cloned in derivatives of pBR322), using convenient restriction sites, and ultimately into pAd2BsuA (the *Bsu*36I A fragment of Ad2 DNA [nucleotides 7616 to 25986], made blunt ended and ligated between the *Eco*RV and *Pvu*II sites of pBR322). 293 cells were transfected by the calcium phosphate method (15) with Ad2 DNA-terminal protein complexes that were digested with *Bam*HI and a fivefold molar excess of the mutated pAd2BsuA derivatives that were linearized at a unique *Ase*I site in the pBR322 sequence. Recombinant viruses were plaque purified two times, and the mutations were confirmed by restriction enzyme analysis of viral DNA.

Expression of penton base in *Escherichia coli*. The penton base gene was isolated from Ad2 DNA by the polymerase chain reaction, using a sense-strand primer that introduced an *Nhe*I site upstream of the initiator ATG codon (underlined), 5′-TTTTGCTAGCATGCAGCGCGCGG, and an antisense strand primer that annealed 10 nucleotides beyond the terminator codon and introduced a downstream *Not*I site, 5′-GGGAATTCGCGGCCGCGGGCGATATAAGGATGG. Initially, the *Nhe*I-*Not*I fragment was cloned into a derivative of the baculovirus shuttle vector pJVP10-Z (43), which was modified by insertion of a *Not*I restriction site (37). Subsequently a portion of the insert, extending from the *Nhe*I site to an internal *Bam*HI site at Ad2 nucleotide 15403, was subcloned into the bacteriophage T7-derived expression vector pET11a (38). The gene was completed by inserting into the *Bam*HI site the Ad2 *Bam*HI-*Bgl*II fragment from nucleotides 15403 to 16588. *E. coli* BL21DE3 (38) was transformed to ampicillin resistance by the resulting construct, pET11aPB. Expression of the penton base gene was induced in late-log-phase cultures (optical density at 600 nm of 0.8) in LB broth at 37°C by adjusting the medium to 0.4 mM isopropylthiogalactopyranoside (IPTG). After shaking for 3 h, cells were collected by centrifugation, washed in STE (10 mM Tris-HCl [pH 8], 1 mM EDTA, 100 mM NaCl), lysed by three cycles of rapid freezing and thawing in the presence of 0.1 mg of lysozyme per ml, and sonicated twice for 30 s with a microprobe tip at 40% of maximum output in a Branson Sonifier. The insoluble fraction, which contained penton base, was collected by centrifugation, resuspended by sonication with a cuphorn probe (Branson) into buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% Nonidet P-40), and recentrifuged. After three cycles of washing, the final pellet was dissolved in a small volume of 6 M urea, diluted with 9 volumes of buffer (50 mM K_3PO_4 [pH 10.7], 50 mM NaCl, 1 mM EDTA), and dialyzed against phosphate-buffered saline (PBS) and then 50 mM phosphate buffer (pH 7.5). Protein was sterilized by filtration and stored at 4°C or frozen at −70°C. To construct deletion mutants, pET11aPB was cut at a unique *Eco*RI site in vector DNA and at several different restriction sites within the penton base gene indicated in Table 1. The DNA was made blunt ended by using the Klenow fragment of DNA polymerase I and then ligated with T4 DNA ligase. Termination codons occur in all three reading frames just beyond the *Eco*RI site.

Cell rounding and adhesion assays. Confluent monolayers of A549 cells in 24-well dishes (Costar) were washed with PBS, and then 0.5 ml of DME without serum was added to each well. Proteins in phosphate buffer were added in a volume of 5 to 20 μl per well; the plate was incubated for 1 to 2 h at 37°C in a 5% CO_2 atmosphere and then examined microscopically to score for cell rounding. For cell adhesion assays, Immunlon 2 96-well plates (Dynatech) were coated overnight at 4°C with 100 μl of protein dissolved in 6 M urea. Protein solutions were removed, and wells were blocked for 2 h at room temperature with 200 μl of 2% bovine serum albumin (BSA) in PBS. Wells were rinsed three times with PBS containing 0.1% Tween 20, and then 100 μl of DME without serum was added per well. Competing proteins, when required, were added at this stage; then 4×10^4 HeLa S3 cells were added in 10 μl of DME, and the plate was incubated at 37°C in an atmosphere of 5% CO_2 for 1 h. Wells were washed two times with PBS to remove unattached cells. Adherent cells were fixed for 30 min at room temperature with 4% paraformaldehyde in PBS and then stained for 15 min with 1% toluidine blue in 4% paraformaldehyde–PBS. Plates were washed with PBS until supernatants were clear.

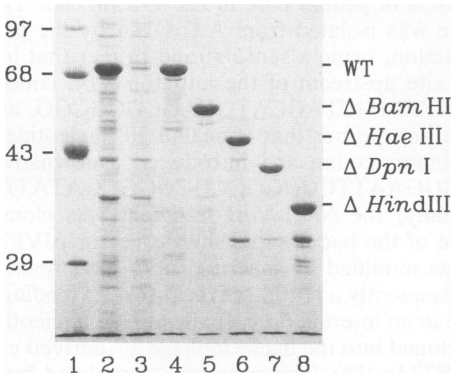

FIG. 2. SDS-polyacrylamide gel electrophoresis of Ad2 penton base and deletion mutants expressed in *E. coli*. Lanes: 1, molecular weight standards; 2 to 4, whole cells and soluble and insoluble fractions of cell lysates expressing full-length penton base; 5 to 8, insoluble fractions of lysates of cells expressing Δ*Bam*HI, Δ*Hae*III, Δ*Dpn*I, and Δ*Hin*dIII deletion mutants (see Table 1). Sizes are indicated in kilodaltons. WT, wild type.

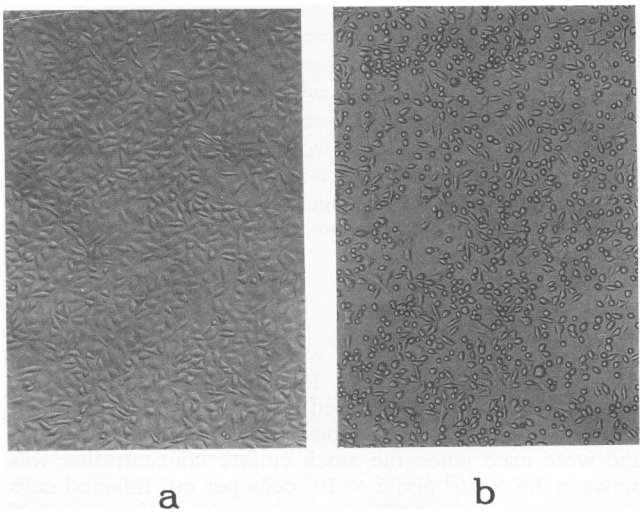

FIG. 3. Effect of recombinant penton base on A549 cell morphology. (a) Untreated cells; (b) cells exposed to recombinant penton base (3 μg/ml) for 1 h.

Cell-associated stain was extracted for 15 min with 200 μl of methanol per well, and the A_{625} was read in a spectrophotometer.

RESULTS

Expression of Ad2 penton base in *E. coli*. The penton base gene was amplified from the Ad2 genome by the polymerase chain reaction and was cloned in the bacteriophage T7-derived expression vector pET11a (38). In the resulting construct (pET11a-PB), the penton base reading frame begins in vector DNA, which encodes a four-residue extension, Met-Ala-Ser-Thr-, at the N-terminal end of penton base. Penton base was overexpressed in *E. coli* cells transformed by pET11a-PB and was found in the insoluble fraction of cell lysates (Fig. 2, lanes 2 to 4). Inclusion bodies containing penton base were washed extensively with nonionic detergent and dissolved in 6 M urea. After dialysis against phosphate buffer and centrifugation to remove precipitates, a soluble preparation of recombinant penton base was obtained. Amino acid sequence analysis showed Ala-Ser-Thr in the first three cycles, confirming the presence of the T7-encoded N-terminal extension.

Mutant analysis of the penton base cell rounding activity. Addition of recombinant wild-type penton base (3 μg/ml) to the medium of A549 cell monolayers caused a marked rounding of cells within 1 to 2 h (Fig. 3); this effect was abolished by digestion of the penton base preparation with trypsin. Truncated versions of penton base were constructed by deleting different restriction fragments from pET11a-PB. All constructs were overexpressed (Fig. 2, lanes 5 to 8), and refolded proteins were prepared according to the same protocol that was used for the full-length penton base. Cell rounding activity was detected in all derivatives that extended beyond amino acid 354 but was not detected in shorter polypeptides that terminated at amino acid 295 or 336 (Table 1).

Amino acids 337 to 353 contain the sequence Arg-Gly-Asp (residues 340 to 342). This sequence also occurs in proteins that form the extracellular matrix (ECM), where it is recognized by receptors in the integrin family (27, 30). Exposure of adherent cells to RGD-containing peptides displaces integrins from the ECM, causing cells to round up and detach (16). To test whether the penton base cell rounding activity also occurs by a similar mechanism, four mutants were constructed in which the penton base RGD sequence was converted to RAE, RGE, SGD, or RVD. As shown in Table 1, none of these mutants was active in the cell rounding assay. Surprisingly, the deletion mutant RGDTFA, which is truncated three residues after the RGD sequence and was constructed by introduction of a stop codon, also was inactive in the cell rounding assay but had partial activity in another assay (see below).

Cells bind to the penton base RGD motif. To demonstrate that cell rounding results from binding of cells to penton base, we used an assay that was developed for studying adhesion of cells to proteins in the ECM (29). Untreated polystyrene (petri) wells were coated with proteins and then seeded with HeLa S3 cells from suspension culture. After a 1-h incubation at 37°C, wells were rinsed to remove unattached cells, the attached cells were stained with toluidine blue, and the absorbance was determined to estimate cell number. The wild-type penton base was strongly active, retaining most of the cells applied to the wells, but the four point mutants with altered RGD sequences were not active

TABLE 1. Cell rounding activity of penton base deletion and point mutants

Protein[a]	Activity
Wild type (1–571)	+
Δ*Bam*HI (1–419)	+
Δ*Hae*III (1–354)	+
Δ*Dpn*I (1–336)	−
Δ*Hin*dIII (1–295)	−
RGE [D342E, T343S]	−
RVD [G341V]	−
SGD [I339L, R340S]	−
RAE [G341A, D342E]	−
RGDTFA (1–345)	−

[a] Penton base amino acids present in deletion mutants are indicated in parentheses; amino acid substitutions are indicated in brackets.

TABLE 2. Binding of HeLa S3 cells to protein-coated wells

Protein on matrix	Inhibitor[a]	Optical density units/well[b]
rPB[c]		0.69 ± 0.06
	BSA	0.70 ± 0.05
	Fiber	0.66 ± 0.06
	Penton	0.17 ± 0.02
	rPB	0.10 ± 0.01
	RVD	0.59 ± 0.01
Penton		0.84 ± 0.01
RGDTFA		0.33 ± 0.01
RAE		0.05 ± 0.01
RGE		0.04 ± 0.01
SGD		0.04 ± 0.01
RVD		0.04 ± 0.01
BSA		0.04 ± 0.01

[a] Inhibitor stock solutions were used at concentrations of between 0.5 and 1 mg/ml and were present in assay medium at 10% (vol/vol).

[b] Attached cells were stained with toluidine blue, and the A_{625} was measured (mean of duplicate wells ± standard deviation). The number of residual cells per well was estimated microscopically to confirm that absorbance was proportional to cell number.

[c] rPB, recombinant penton base.

and did not retain cells above the background levels observed with the negative control protein BSA (Table 2). Deletion mutant RGDTFA was partially active in this assay. The binding of inactive penton base proteins to polystyrene wells was confirmed by an enzyme-linked immunosorbent assay (not shown) using antiserum raised against recombinant penton base. Cell attachment to the recombinant penton base was blocked by addition of native pentons or recombinant wild-type penton base to the plating medium but was not blocked by purified fiber or the recombinant RVD mutant protein.

RGD functions early during infection. To examine penton base RGD function in the context of virus infection, we constructed viruses that contained the RAE, RGE, RVD, and SGD mutations described above. These virus mutants were isolated from 293 cells that were transfected with *Bam*HI-digested Ad2 DNA and the Ad2 *Bsu*36I A fragment (nucleotides 7616 to 25986) which was cloned in pBR322 (pAd2BsuA) and contained the mutated penton base genes. Homologous recombination between pAd2BsuA and the terminal *Bam*HI fragments generated infectious viral genomes carrying the penton base mutations, as was previously described for other viral DNA fragments (4, 44). Virus mutants were recovered in all four cases, and the mutations were confirmed by restriction enzyme analysis (not shown). Examination of the kinetics of virus production at 37°C in A549 cells (Fig. 4a) showed that all four mutants remained in the eclipse period 5 to 10 h longer than the wild-type virus; however, the slope of the log phase and the final yield of virus per cell were similar to values for wild-type Ad2. A prolonged eclipse period also was observed during mutant infections carried out at 32°C (not shown). Mutant virions purified on CsCl gradients were similar to wild-type Ad2 with respect to both infectivity (particle/PFU ratio) and heat stability (Fig. 5).

An extended eclipse period could reflect a malfunction either of the penton base associated with the virus inoculum or of the penton base that is synthesized during infection. Since penton base is synthesized during the late phase of infection, after the onset of viral DNA synthesis, early or late effects of the mutations can be determined by examining the kinetics of mutant DNA synthesis. A549 cell monolayers were infected with mutant or wild-type virus, and viral DNA was recovered by Hirt extraction at 2-h intervals from 12 to 20 h postinfection and analyzed on agarose gels (Fig. 6a; results for all mutants were similar; only data for the RGE mutant are shown). Viral DNA in wild-type-infected cell extracts was visible by ethidium bromide staining at 12 h postinfection, indicating that substantial replication of the viral genome had occurred. Mutant DNA was not detected until 18 h postinfection, indicating a delay in the onset of mutant DNA synthesis. Therefore, the observed delay in reproduction of virus mutants in A549 cells results from the loss of an early function associated with the infecting virions, not from defective folding or assembly of newly syn-

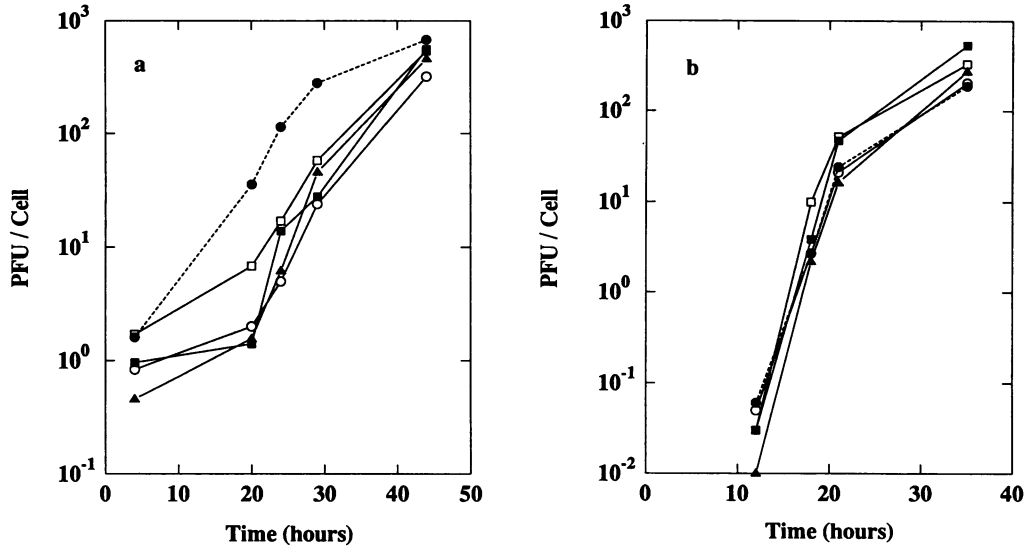

FIG. 4. Reproduction of wild-type and RGD mutant viruses in A549 and HeLa S3 cells at 37°C. Cells were infected at a multiplicity of 10 PFU per cell; at the indicated times, the virus yield was quantitated by indirect immunoassay. (a) A549 cells; (b) HeLa S3 cells. Symbols: ●, WT; ■, SGD; □, RVD; ▲, RGE; ○, RAE.

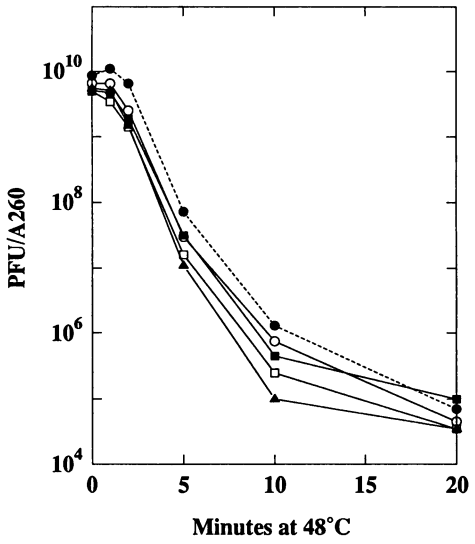

FIG. 5. Heat inactivation of virus stocks. CsCl-purified wild-type and RGD mutant viruses were diluted to 0.1 A_{260} in Tris-buffered saline and placed in a 48°C water bath. Samples were removed at the indicated times, and virus infectivity was quantitated by indirect immunoassay. Symbols: ●, WT; ○, RAE; ■, SGD; □, RVD; ▲, RGE.

thesized penton base. This conclusion is consistent with data in Fig. 4a, which show that once the virus mutants emerge from eclipse, they assemble new infectious particles at a rate and with a final yield similar to those for wild-type Ad2.

Virus reproduction in nonadherent cells. In contrast to the delay in mutant virus reproduction observed in A549 cells, the time course of infection of HeLa S3 suspension cell cultures by mutant virus was indistinguishable from that of wild-type Ad2, as seen in growth curves (Fig. 4b) and in the

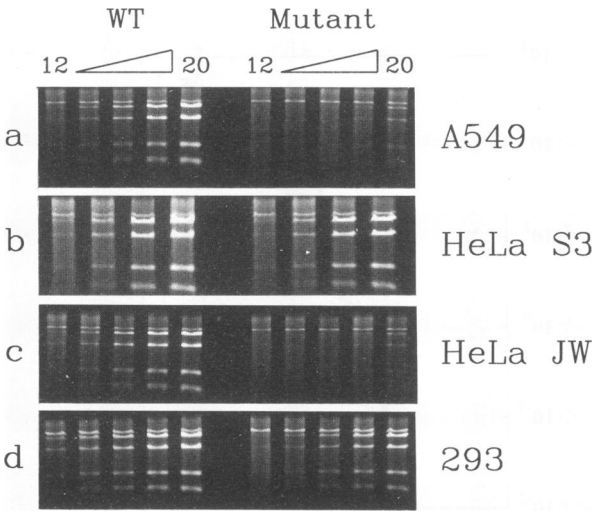

FIG. 6. Viral DNA synthesis in adherent and nonadherent cells. Cells were infected with wild-type (WT) and RGE mutant viruses at a multiplicity of 10 PFU per cell. Samples were taken between 12 and 20 h postinfection, and viral DNA in Hirt supernatants was digested with *Bam*HI and electrophoresed in 0.8% agarose gels. (a) A549 cells; (b) HeLa S3 cells; (c) HeLa JW cells; (d) 293 cells.

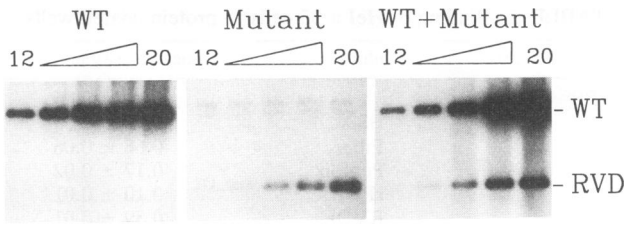

FIG. 7. Complementation analysis of the RVD mutant. Viral DNA was isolated from A549 cells that were singly or doubly infected with wild-type (WT) and RVD mutant viruses and was digested with *Sal*I and analyzed by Southern blotting. The hybridization probe, the Ad2 *Bam*HI-*Sal*I fragment from nucleotides 15403 to 16744, is contained entirely within a single *Sal*I fragment from each viral DNA (the RVD mutation introduces a new *Sal*I site into the Ad2 genome at nucleotide 15171).

kinetics of virus DNA synthesis (Fig. 6b). These results suggested that expression of the mutant phenotype might require adherent cells. To test this hypothesis, we plated HeLa S3 cells on tissue culture dishes; however, the cells did not adhere tightly and detached completely during adsorption of the virus inoculum. This problem was circumvented by plating cells on petri dishes that were precoated with penton base. The cells adhered to this surface but remained rounded and did not spread. Mutant and wild-type viral DNA synthesis proceeded with similar kinetics in these cells (not shown). Two HeLa cell subclones which do adhere to tissue culture plastic and form typical, well-spread monolayers were obtained. The onset of mutant DNA synthesis was delayed in these cells by about 6 h (Fig. 6c; results from only one subclone are shown), similar to the kinetics observed in A549 cells (Fig. 6a).

Interestingly, the kinetics of wild-type and RGE mutant viral DNA synthesis were similar in 293 cells, in which the accumulation of mutant DNA was delayed about 2 h in the experiment shown (Fig. 6d). In contrast to the other adherent cells used in this study, 293 cells have a rounded or spindle-shaped morphology and are easily detached by pipetting. Plating 293 cells on penton base-coated petri dishes did not alter their morphology and had no effect on the kinetics of DNA synthesis of the mutant viruses (not shown). Another feature of 293 cells is their constitutive expression of the adenovirus E1 transcription unit (1, 14). 293 cells contain levels of the E1a-encoded transcription transactivators sufficient to complement E1a-null virus mutants (17). To determine whether the penton base mutants could be complemented by E1a in *trans*, the kinetics of viral DNA synthesis were examined in coinfections of A549 cells with wild-type Ad2 and the RVD penton base mutant. The RVD mutation introduces a novel *Sal*I restriction site into the viral genome, permitting the wild-type and mutant genomes to be distinguished. A549 cells were singly or doubly infected, and viral DNA was recovered at 2-h intervals from 12 to 20 h postinfection, digested with *Sal*I, and analyzed on Southern blots (Fig. 7). The onset of mutant DNA synthesis occurred during this interval, as shown by the increase in DNA levels between 14 and 16 h. Wild-type DNA replication was nearly complete at 16 h, however, as indicated by similar levels of DNA at 16, 18, and 20 h postinfection. In coinfections, the onset of mutant DNA synthesis also occurred between 14 and 16 h, indicating that the kinetic defect was not complemented.

Conservation of the RGD sequence. The GenBank data base contains the penton base gene sequence from human

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  2Hu (283) IPALL DVDAY QASLK DDTEQ GGDGA GGGNN SGSGA
 12Hu (273) IPALL DVKKY ENSLQ DQNT- ----- ----- -----
 40Hu (279) IPALL DVEKY EASIK EAQE- ----- ----- -----
FAV-10 (306) IPALL DLDSV DVNDA DGEV- ----- ----- -----

  2Hu EENSN AAAAA MQPVE DMNDH AIRGD TFATR AEEKR AEAEA
 12Hu ----- ----- ----- ----- -VRGD NF--- ----- -----
 40Hu ----- ----- ----- ----- -IRGA DF--- ----- -----
FAV-10 ----- ----- ----- ----- -IELD NAA-- ----- -----

  2Hu AAEAA APAAQ PEVEK PQKKP VIKPL TEDSK KRSYN (392)
 12Hu --IAL NKAAR ----- ----- -IEPV ETDPK GRSYN (319)
 40Hu ----- ----- --KPN PQDLE IV-PV EKDSK ERSYN (325)
FAV-10 ----- ----- ----- ----- ---PL LHDSA GVSYN (343)
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FIG. 8. Alignment of penton base variable domain sequences from different adenoviruses. Conserved residues are indicated in boldface; missing residues are indicated by dashes. The residue numbers of the first and last amino acids included in the alignment are indicated in parentheses. 2Hu, 12Hu, and 40Hu, human Ad2, Ad12, and Ad40; FAV-10, fowl Ad10.

Ad40; the fowl serotype 10 (FAV-10) penton base sequence also was recently reported (36). The protein sequences were manually aligned with the Ad2 sequence. Both ends of penton base are highly conserved. Amino acids 1 to 282 and 393 to 571 of Ad2 are 66 and 81% identical to the corresponding regions of Ad40 penton base and are 47 and 41% identical to the corresponding regions of the FAV-10 protein. Most amino acid substitutions in these regions are conservative. The central region, however, is poorly conserved, and both Ad40 and FAV-10 have deletions of more than 60 amino acids relative to Ad2 (Fig. 8). Strikingly, these deletions occur in two nearly equal-size blocks that are separated by the RGD sequence, which itself is not strictly conserved in either Ad40 or FAV-10. Recently we reported that the penton base from human Ad12 has a substantially smaller apparent molecular weight than the Ad2 protein, based on its mobility in SDS-polyacrylamide gels (11). It was therefore of interest to examine the central region for deletions and for conservation of the RGD sequence. A fragment of the Ad12 genome encoding this region was cloned and sequenced (to be reported elsewhere). The predicted amino acid sequence of the central variable domain is included in the alignment shown in Fig. 8. Like Ad40 and FAV-10, Ad12 has two large deletions in a hyphenated arrangement, but despite radical divergence from the Ad2 sequence, the RGD sequence is conserved in Ad12.

DISCUSSION

In this report, we showed that an RGD sequence was necessary for the cell rounding and cell binding activities of the Ad2 penton base protein that was expressed in bacteria. RGD-containing proteins from the ECM have similar effects on cell adhesion, resulting from specific recognition of the RGD sequence by cell adhesion receptors in the integrin family (27, 30). Therefore, it seems likely that the RGD sequence in penton base also is recognized by an integrin. This interaction probably accounts for the cytopathic effect of penton base noted in earlier studies (9, 22, 28). Unassembled pentons accumulate to high concentrations in cultured cells, raising the question of whether pentons have a physiological effect in human tissues. Release of unassembled pentons at the locus of infection might have effects on cell adhesion in surrounding tissues or on the adhesive activities of lymphocytes or other cells that are recruited to the site of infection. Evaluation of the physiological effects of this protein should accompany the development of adenovirus-based vaccines or vectors for use in gene therapy.

Alignment of the available penton base sequences showed that the RGD sequence occurs in a hypervariable region of the protein and is not strictly conserved in Ad40 or FAV-10. DNA sequence analysis of the corresponding region from Ad12 showed similar variations in amino acid sequence and polypeptide chain length; however, it was striking that the RGD sequence is conserved in Ad12 (Fig. 8). The extreme variation in sequence suggests this region is likely to have a serotype-specific function. It is interesting that the other component of the capsid vertex, the penton fiber, also varies markedly in length among different adenovirus serotypes (24) and that fiber receptors may be serotype or subgroup specific (8).

The sequence alignment also suggests that penton base contains two structural domains which are relatively invariant and therefore probably mediate conserved functions such as the association of penton base into pentamers, its assembly with fiber subunits, and the assembly of pentons into the vertex of the capsid. Complementation analysis between temperature-sensitive mutants of human Ad5 and Ad7 showed that Ad7 fibers can assemble on Ad5 penton bases (26), indicating that the fiber binding site in penton base is conserved. Considering the probable structural constraints on penton base, it seems likely that the hypervariable central region occupies a position independent of the conserved domains, where variations in the polypeptide chain length could be accommodated without disturbing contacts between penton base and other structural proteins of the virion. Location of the RGD-containing region on the exterior surface of the capsid would be necessary if virions interact with adhesion receptors during virus uptake.

The hypervariable central domain in Ad2 is predicted to contain two long α helices connected by the RGD sequence in a β turn (5, 12). Interaction between these helices might stabilize the RGD sequence in an active conformation, accounting for the potent cell rounding activity of penton base. This might explain why the deletion mutant RGDTFA, which is truncated three residues downstream of the RGD sequence, was inactive in the cell rounding assay and only weakly active in the cell adhesion assay; sufficient sequence to form the downstream helix is not present in this mutant. The predicted secondary structure of the Ad2 penton base hypervariable domain bears little resemblance to the structures of the RGD-containing domains of tenascin (19) and fibronectin (20), which contain two β sheets in an immunoglobulin-related fold. However, in tenascin and fibronectin, the RGD sequence occurs in a loop, similar to its predicted secondary structure in penton base. This loop is shorter in tenascin and was suggested to account for why the fibronectin receptor cannot bind the tenascin RGD (19). It is tempting to speculate that the polypeptide chain length variations in the penton base central region also may reflect usage of alternate receptors and that a functional ligand at this position may be a conserved feature in all adenoviruses, e.g., that cognate receptors may exist for the Ad40 and FAV-10 penton bases.

The role of the penton base RGD sequence in the context

of virus infection is not clear. It is possible that the RGD sequence has no interaction with host cell molecules and that the amino acid substitutions that we constructed have only structural consequences that might affect capsid disassembly, for example. Our results showing that all four mutants had normal particle/PFU ratios is consistent with the hypothesis that the RGD sequence has no role in virus adsorption or uptake. However, we could not detect a significant difference in the heat inactivation kinetics of mutant and wild-type viruses. Furthermore, none of the viruses was temperature sensitive for reproduction (not shown), and the rate of mutant virus production and the final yield of virus per cell were similar to those for wild-type virus in all cases, suggesting that structural damage to the capsid was minimal. It is possible, but unlikely, that the structural consequences of four different RGD mutations depend on a cellular feature that varies between the cell lines used in this study.

Expression of the mutant phenotype exclusively in the flat cells and not in any of the round cells that we tested is indirect evidence for an interaction between the virion penton base and an adhesion molecule. Assuming that such an interaction does occur, then it might affect the binding of virus to the cell surface or a subsequent intracellular event. As noted above, loss of the putative ligand does not affect the particle/PFU ratio, arguing against a role for the RGD sequence in virion adsorption. Signaling through an integrin also seems unlikely, since the RVD mutant was not complemented by wild-type Ad2 in coinfections. Indeed, the complementation analysis suggests that the mutant and wild-type viruses are sequestered in separate compartments during the critical period. This may correspond to the period when virus is within intracellular vesicles, possibly endosomes or lysosomes. In round cells (HeLa S3 and 293), the adhesion molecules, if they are expressed, are likely to be diffusely distributed over the cell surface rather than funneled to the basal surface as on flat cells. This could permit cointernalization of virus and receptor in the absence of an RGD ligand. The receptor might indirectly influence the fate of virus in endosomes, perhaps by regulating ion fluxes across the endosomal membrane as it does across the plasma membrane (31, 32). Our data do not resolve whether the missing or altered function of the mutants arises from an inability to cointernalize an adhesion molecule with the virus or from a structural alteration of the mutant capsid.

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ADDENDUM

Recently we observed that the virus mutants adsorbed to cells at 4°C become resistant to neutralizing antihexon antibodies at a slower rate after shifting to 37°C than does the wild-type virus, suggesting that interaction of the virion RGD sequence with an adhesion receptor accelerates the endocytosis of virus-receptor complexes.

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