Mutations in the carboxy terminus of adeno-associated virus 2 capsid proteins affect viral infectivity: lack of an RGD integrin-binding motif

Michael Ruffing,† Hans Heid and Jürgen A. Kleinschmidt

1 Deutsches Krebsforschungszentrum, Forschungsschwerpunkt Angewandte Tumorvirologie and 2 Forschungsschwerpunkt Krebsentstehung und Differenzierung, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

Using site-directed mutagenesis, we tested whether a potential integrin-binding site, (composed of the amino acids RGD) which is predicted in the adeno-associated virus 2 (AAV-2) capsid open reading frame (ORF), plays a role in the infectivity of AAV-2. Nucleotide sequencing of wild-type and mutant capsid protein-coding sequences, however, revealed discrepancies with the published sequence data at several positions, including a frameshift in the carboxy terminus which cancels the RGD motif and extends the capsid ORF by 27 amino acids. This sequence was confirmed by protein sequencing of proteolytic fragments of VP3. Thus, the virus mutant (pTAV-p), in which the intention was to exchange D of the putative RGD motif for E, resulted in replacing 1480 by S in the newly established ORF. A second virus mutant (pTAV-d), in which the intention was to delete the RGD peptide, in fact gave a shift into the ORF of the originally published sequence. The pTAV-p mutant showed a strongly reduced infectivity compared to wild-type AAV-2, whereas pTAV-d was not infectious at all. Neither mutant accumulated viral ssDNA as detected by Hirt extraction. Analysis of virus particle formation and subcellular localization of the capsid proteins revealed a defect of the mutant capsid proteins in capsid assembly. This shows that the newly established C-terminal sequence of the AAV capsid proteins plays an important role in viral assembly.

Introduction

The adeno-associated virus 2 (AAV-2) is a human parvovirus that needs a helper virus for efficient replication (for reviews see Berns, 1990; Berns & Bohensky, 1987; Muzychka, 1992). The viral genome encodes four non-structural proteins, termed Rep proteins, which are needed for control of gene expression and DNA replication and are encoded by an open reading frame (ORF) on the left side of the genome (Hermonat et al., 1984; Mendelson et al., 1986; Srivastava et al., 1983; Tratschin et al., 1984). The three capsid proteins (VP1, VP2 and VP3) are encoded by another ORF located on the right side of the genome (Becerra et al., 1985, 1988; Cassinotti et al., 1988; Janik et al., 1984; Srivastava et al., 1983). The structural proteins form an icosahedral capsid 20 to 24 nm in diameter with a stoichiometry of 1:1:10 for VP1:VP2:VP3. They are expressed from different translation initiation codons of the same ORF. The observed stoichiometry of 1:1:10 is generated by the relative abundance of an alternatively spliced mRNA, from which VP1 is translated, and a reduced translation initiation frequency of VP2 from an unusual initiation codon (ACG) at position 2615 (Muralidhar et al., 1994). Assembly studies suggest a two-step assembly process: first empty capsids are formed then the ssDNA virus genome is packaged in these to form mature particles (Myers & Carter, 1980). It is assumed that capsid formation is a prerequisite for the accumulation of ssDNA (Myers & Carter, 1981). Mutations in the VP initiation sites have shown that VP2 and VP3 are sufficient and necessary for the accumulation of single-stranded progeny DNA, whereas VP1 seems to be required for production of infectious particles (Smuda & Carter, 1991; Muralidhar et al., 1994). In addition, besides the two large replication proteins (Rep78 and Rep68) at least one of the small Rep proteins (Rep52 or Rep40) is also required for ssDNA accumulation (Chejanovsky & Carter, 1989).

AAV-2 can infect a wide variety of cell types and seems to multiply productively in any mammalian cell line that can be infected by a helper virus (Muzychka, 1992). The cellular receptor(s) to which AAV binds has not been identified yet. According to the nucleotide sequence published by Srivastava et al. (1983) the amino
acid sequence of the three capsid proteins should include
an RGD peptide within the carboxy-terminal part. This
motif has been identified in numerous proteins and is
involved in adhesion to cellular receptors classified as
members of the integrin receptor family (Hynes, 1992;
Ruoslathi & Pierbacher, 1986, 1987). RGD motif-
containing proteins include the capsid proteins of foot-
and-mouth-disease virus (Fox et al., 1989) and coxsackie-
virus A9 (Roivainen et al., 1991). Using site-directed
mutagenesis we set out to test whether this motif exerts a
similar function in the AAV-2 capsid proteins.

The construction of these mutants was based on the
previously published AAV-2 sequence (Srivastava et al.,
1983). Analysis of the resultant plasmids showed that the
original sequence was incorrect and that the C terminus
extended another 27 amino acids due to frameshifting to
an ORF that does not contain the RGD motif. Thus, the
mutant plasmids coded for mutant proteins different
from those originally anticipated. The phenotype of
these mutants suggests that the newly established C
terminus of the capsid proteins plays an important role
in AAV assembly.

Methods

Transfection of HeLa cells and generation of virus stocks. Transfection
of HeLa cells was performed according to Chen & Okayama (1987).
For preparation of wild-type and mutant virus stocks 2 × 106 cells were
transfected with 15 μg pTAV-2 (Heilbronn et al., 1990), pTAV-p
or pTAV-d, respectively, infected with adenovirus type 2 (m.o.i. of 5)
and collected from the culture medium 4 to 5 days post-infection.

Mutagenesis and construction of virus mutants. The 2.9 kb HindIII/ 
SrfI fragment comprising the complete sequence encoding the capsid
protein was isolated from the plasmid pTAV-2 (Heilbronn et al., 1990)
and cloned into the vector M13mp18. The resulting plasmid mp18-
2.9HS was used for site-directed mutagenesis (Taylor et al., 1985) using
an Amersham kit following the supplier’s instructions. Mutagenesis
was performed using the oligonucleotide 5′-GGTCAGCGTTGAGA-
GCGAGTGAGGAGCT-3′ (position 4228 to 4254) to replace nucleotide
T at position 4242 [sequence position according to Srivastava et al.
(1983) and Cassinotti et al. (1988)] by G for generation of plasmid
pTAV-p and using the oligonucleotide 5′-ACGGGACACGGTCAGC-
GAGTGGGAGCTGCA-3′ (position 4219 to 4257) to delete the
nucleotides from position 4233 to 4242 for generation of plasmid
pTAV-d. Mutants were identified by DNA sequencing according to the
method of Sanger et al. (1977). A 0.3 kb fragment resulting from
restriction of the mutated plasmids with BspM1 and SnaB1 was
isolated and cloned into pTAV-2 replacing the wild-type sequence.
These plasmids were sequenced and used for the production of virus
stocks.

DNA and protein sequence analysis. Nucleotide sequencing of
plasmids pTAV-2 (Heilbronn et al., 1990; Laughlin et al., 1983) and
pSM620 (Samulski et al., 1982) was performed according to Sanger et
al. (1977) using synthetic oligonucleotide primers from the VP3 ORF.
Protein sequences of peptides were obtained by digestion of baculo-
virus-expressed VP3 protein (Ruffing et al., 1992) with endoproteinase
lys C. Briefly, 102 Sf9 (Spodoptera frugiperda) cells were infected with
VP3-recombinant baculoviruses and lysed by sonication (five pulses at
power position 6 of a Branson sonifier for 10 seconds, with 50 seconds
intervals in ice water) 60 to 72 h later in a buffer containing 1 % NP40,
150 mm-NaCl, 20 mm-Tris–HCl (pH 8), 10 mm-DTT and 5 mm-EDTA.
After sedimentation by centrifugation (15 min, 12000 g at 4 °C) the
pellet was resuspended in 8 mm-sucrose, 20 mm-Tris–HCl (pH 7.5) and
incubated for 1 h at room temperature. After centrifugation (15 min,
12000 g at 20 °C) the supernatant was separated by SDS-PAGE and
transferred to an Immobilon-P membrane (Millipore). The position of
VP3 was visualized by staining with Poncet S. The corresponding
region was excised and incubated in a buffer containing 200 mm-
Tris–HCl (pH 8), 0.2% Tween-20 and 5 mm-EDTA for 1 h at room
temperature. After washing the membrane pieces in distilled water,
the polypeptides were digested with endoproteinase lys C in a buffer
containing 5 m-guanidinium-HCl, 200 mm-Tris–HCl (pH 8) and 5 mm-
DTT for 24 h at room temperature and then 2 h at 37 °C. Trifluoroacetic
acid was added to a final concentration of 10 %. The peptides were
purified by HPLC (Applied Biosystems, model 130A; column:
Brownlee-C4; length, 100 mm; diameter, 2.1 mm) and sequenced by
Edmann degradation (Applied Biosystems, protein sequencer 477A).

Analysis of protein expression. For analysis of protein expression by
gel electrophoresis and immunoblotting we prepared total lysates of
HeLa cells transfected with pTAV-2, pTAV-p, or pTAV-d, respectively,
and infected with adenovirus. Cells were harvested 24 h post-infection
and lysed by heating at 100 °C for 5 min in 150 μl sample buffer
(Laemmli et al., 1970). After sonication, polypeptides were analysed by
SDS–PAGE (Thomas & Kornberg, 1975) and immunoblotting (Har-
low & Lane, 1988) using an anti-VP rabbit serum (Ruffing et al., 1992;
1:2000) which recognizes all three capsid proteins, mixed (1:5) with
a monoclonal antibody (A69) which recognizes VP1 and VP2 only, to
increase the sensitivity for VP1 and VP2 detection. Capsid proteins
were visualized by peroxidase-conjugated secondary antibodies and
enhanced chemiluminescence detection (Amersham).

For the detection of capsid formation, freeze-thaw lysates of 2 × 106
HeLa cells transfected with pTAV-2, pTAV-p or pTAV-d, respectively,
and infected with adenovirus, were sedimented by high-speed centri-
fugation (3 h, 200000 g at 4 °C; Sorvall TH641 rotor). The sediments
were analysed by gel electrophoresis and Western blotting.

Analysis of DNA replication. Hirt extracts of HeLa cells transfected
with pTAV-2, pTAV-p, or pTAV-d, respectively, and infected by
adenovirus-2 were prepared according to Redemann et al. (1989) and
analysed by Southern blotting using an Avu I fragment of 1970 bp
comprising the rep ORF.

Immunofluorescence analysis. For immunofluorescence, cells were
grown on coverslips and transfected as described above. Immunofluo-
rescence staining was performed essentially as described by Ruffing
et al. (1992). In addition to the anti-VP3 serum we used the monoclonal
antibody A69 which recognizes VP1 and VP2 only. For double
immunofluorescence, undiluted hybridoma supernatants of A69 were
mixed with an equal volume of anti-VP3 serum (diluted 1:50 in PBS).
Bound antibodies from the VP3 serum were visualized by rhodamine-
conjugated secondary antibodies and bound A69 antibodies by
fluorescein isothiocyanate-conjugated secondary antibodies.

Results

Generation of viral mutants with alterations of the
putative RGD integrin-binding motif and correction of
the AAV capsid protein sequence

Changing the amino acid D of the RGD motif into E has
been reported to significantly reduce binding of peptides
to the integrin receptors of a variety of cells, (Russo...
AAV-2 genome •

Fig. 1. Intended mutagenesis of the putative RGD peptide in the carboxy-terminal part of the AAV capsid proteins. The AAV-2 genome is depicted, with the inverted terminal repeats indicated by filled rectangular boxes, the three promoters (p) at map unit positions 5, 19 and 40, and the region coding for the capsid proteins (VP) indicated by arrows. Below, the putative RGD peptide of the wild-type genome present in plasmid pTAV-2 and the intended changes in pTAV-p and pTAV-d mutants are shown. The single letters indicate restriction sites used for the generation of the VP mutants: H, HindIII; B, BssHII; S, SnaBI and Ss, SstI. The numbers indicate nucleotide positions, according to Srivastava et al. (1983) and Cassinotti et al. (1988).

& Piersbacher, 1986, 1987). To create this amino acid exchange in the AAV VP-coding sequence, nucleotide 4242 [according to Srivastava et al. (1983) and corrected by Cassinotti et al. (1988)] was changed from T to G by oligonucleotide-directed mutagenesis. The resultant mutated AAV plasmid was designated pTAV-p ('p' for point mutated). The same method was used to generate the plasmid pTAV-d ('d' for deleted) which lacks the sequence from nucleotide 4233 to 4242 which is supposed to code for the RGD peptide (Fig. 1). Nucleotide sequencing of the VP-coding region of the mutant plasmids and the two wild-type plasmids pTAV-2 (Heilbronn et al., 1990; Laughlin et al., 1983) and pSM620 (Samulski et al., 1982) showed that the published data have to be corrected at several positions (see Table 1). In the amino-terminal part of the shortest capsid protein, VP3, the following corrections have to be made in the coding strand: replace nucleotide C at position 2877 by G; insert the sequence GGCCCG after nucleotide position 3759; replace A at position 3853 by G; insert A after nucleotide position 3891 and delete A at position 3895 [nucleotide positions are given according to Srivastava et al. (1983), and corrected by Cassinotti et al. (1988)].

Correction of the nucleotide sequence in the carboxy-terminal part includes the deletion of the nucleotide C at position 4227, deletion of C at position 4325, insertion of T after nucleotide 4333 and changes of C (4336) into T and T (4342) into C. The deletion of C at position 4227 results in a frameshift three amino acids upstream of the putative RGD sequence, thus cancelling the RGD motif and extending the capsid protein ORF by 27 amino acids (Fig. 2a, reading frame is underlined). The complete amino acid sequence deduced from the corrected nucleotide sequence is shown in Fig. 2(b). Amino acid changes are shown in bold letters and are labelled with a dot. The predicted change in the ORF was confirmed by sequencing of peptides obtained by digestion of VP3 expressed in insect cells (Fig. 2b, underlined sequences). The baculovirus expression clone of VP3 was derived from pTAV-2 (Heilbronn et al., 1990).

The oligonucleotide used for generation of the pTAV-d mutant was based on the published sequence and overlapped with the sequence corrected at position 4227. Thus it gave rise not only to the deletion of the putative RGD motif, but also to a shift to the ORF of the

Table 1. Sequence corrections to the AAV-2 VP3 ORF

<table>
<thead>
<tr>
<th>Nucleotide position*</th>
<th>Amino acids of the VP3 ORF</th>
<th>Sequence obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change C(2877) to G</td>
<td>No change</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Insert GGC CCG after G(3759)</td>
<td>Insert G and P</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Change A(3853) to G</td>
<td>Change N to D</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Insert A after C(3891)</td>
<td>Change G to R</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Delete A(3895)</td>
<td>No change</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Delete C(4227)</td>
<td>(i) Change H to Q</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td></td>
<td>(ii) Frameshift</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Delete C(4325)</td>
<td>Frameshift</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Insert T after T(4333)</td>
<td>(i) Change L to F</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td></td>
<td>(ii) Frameshift</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Change C(4336) to T</td>
<td>No change</td>
<td>pSM620, pTAV-2</td>
</tr>
<tr>
<td>Change T(4342) to C</td>
<td>No change</td>
<td>pSM620, pTAV-2</td>
</tr>
</tbody>
</table>

* Nucleotide positions are given according to Srivastava et al. (1983) and corrected by Cassinotti et al. (1988).
† Samulski et al. (1982).
‡ Heilbronn et al. (1990).
§ VP3 amino acid sequences were obtained from VP3 expressed in insect cells according to Ruffing et al. (1992).
published nucleotide sequence. This mutation resulted in the truncation of the capsid proteins by 30 amino acids, which should result in a significantly reduced $M_\text{r}$ as observed by gel electrophoresis (see Fig. 4b).

The mutant pTAV-p was generated by an oligonucleotide which did not overlap the sequence corrected at position 4227. However, instead of changing the D of the putative RGD tripeptide to E, I\textsuperscript{490} was replaced by S. Taken together, these results show that the AAV-2 capsid proteins in fact do not contain an RGD peptide sequence and that the observed phenotypes of mutant pTAV-p and pTAV-d are based on genetic alterations that are different from those we intended to introduce by site-directed mutagenesis.

Characterization of infectivity of viral mutants pTAV-p and pTAV-d

In order to compare the infectivity of the viral mutants AAV-p and AAV-d with that of wild-type virus, we prepared wild-type and mutant virus stocks by transfection of HeLa cells with the plasmids pTAV-2, pTAV-p, or pTAV-d, respectively, superinfection with adenovirus-2 and lysis of the cells by freezing and thawing. We then infected HeLa cells in parallel with the respective freeze-thaw supernatants after diluting them to equivalent viral DNA contents (Fig. 3a) and coinfected with adenovirus. Five days after infection cell-free supernatants were prepared and analysed by dot blot hybridization (Fig. 3b).

Limiting dilution of the supernatants showed that the infectivity of AAV-p was reduced about 100-fold compared with that of AAV-2. In the supernatant of pTAV-d-transfected cells no infectivity was detectable. This result was consistent with the assumption that the VP C terminus is important for production of infectious virus.

Expression of the structural and non-structural proteins

To confirm that the AAV non-structural and capsid proteins were expressed in cells transfected by pTAV-p and pTAV-d, respectively, we prepared total cell lysates after superinfection with adenovirus-2 and analysed them by SDS-PAGE and immunoblotting (Fig. 4). The four Rep proteins Rep78, Rep68, Rep52 and Rep40 were detected in equal amounts in cells transfected with
AAV-2 capsid proteins and viral assembly

Fig. 3. Reduced infectivity of the mutants AAV-p and AAV-d compared with the wild-type virus AAV-2. (a) Cells were transfected with pTAV-2, pTAV-p or pTAV-d, respectively, infected with adenovirus and the culture medium was harvested 4 to 5 days post-infection. For subsequent infection experiments the supernatants containing the putative mutant and wild-type virus stocks were appropriately diluted to obtain equivalent amounts of viral DNA as verified by dot blot hybridization. (b) HeLa cells were infected with different dilutions of the supernatants shown in (a) and coinfectected with adenovirus-2. Cell-free lysates were prepared and analysed in a dot-blot assay by hybridization to a radiolabelled NdeI–XbaI fragment of M13mp18–VP3ex (Ruffing et al., 1992).

Fig. 4. Expression of Rep and VP proteins in cells transfected with wild-type and mutant plasmids. Total lysates of HeLa cells transfected with pTAV-2, pTAV-d or pTAV-p, respectively, and infected with helper virus, were analysed by gel electrophoresis and immunoblotting. AAV-2 Rep proteins were detected by the monoclonal antibody 303-9 (a) and the capsid proteins by a mixture of a polyclonal rabbit antiserum and a monoclonal antibody recognizing VP1 and VP2 (b). The specificity of the monoclonal antibody A69 for VP1 and VP2 is shown in (c). Rep and VP proteins were visualized by alkaline phosphatase-conjugated secondary antibodies. Aliquots corresponding to 10^6 cells were used. The correct Mr of expressed Rep and VP proteins was checked by coelectrophoresis of an extract of HeLa cells infected with AAV-2 and adenovirus-2 (not shown).

pTAV-p, pTAV-d, or the control plasmid pTAV-2 (Fig. 4a). Analysis of capsid protein expression also showed that all of the three capsid proteins VP1, VP2, and VP3 were expressed in cells transfected with the mutant or the control AAV plasmids, respectively (Fig. 4b). The greater band intensity of VP1 and VP2 compared with VP3 is due to the enhancement of VP1 and VP2 immunodetection by a VP1/VP2-specific monoclonal antibody (Fig. 4c) which was used together with a polyclonal VP antiserum. Structural proteins extracted from cells transfected with pTAV-p comigrated with the wild-type capsid proteins, but those synthesized by the deletion mutant (pTAV-d) had a significantly reduced Mr, corresponding with the deletion of the 30 amino acids predicted by the corrected VP sequence (Fig. 4b). Taken together, these results mean that the observed phenotypes of the two viral mutants were not due to altered AAV protein expression.

Analysis of ssDNA accumulation and capsid formation

In order to clarify the cause for the reduced infectivity of both viral mutants, we analysed the accumulation of viral ssDNA in HeLa cells after transfection with wild-type and mutant pTAV plasmids and infection with adenovirus-2. In Hirt extracts of cells transfected with pTAV-p or pTAV-d, respectively, no accumulation of AAV ssDNA of defined size could be detected, in contrast to cells transfected with pTAV-2, even after prolonged exposure (Fig. 5a). Replicative forms 1 (RF1) and 2 (RF2) of the viral genome were present in all extracts. This means that the replication of the viral genome is obviously not affected by the mutations. Since infectious lysates could be prepared from cells transfected with pTAV-p (Fig. 3b), we assumed that in this mutant the efficiency of ssDNA synthesis and/or accumulation is reduced to levels that are undetectable in Hirt extracts. The comparable DNA content of pTAV-p and pTAV-d in freeze-thaw supernatants after transfection and infection with helper virus (Fig. 3a) could be due to the transfection input DNA or some release of RF1 and/or RF2 DNA during the freeze-thaw procedure or the cell lysis caused by adenovirus infection.

Since our results did not distinguish whether the low level of ssDNA in Hirt extracts was due to reduced ssDNA synthesis or to less efficient packaging, we analysed the formation of capsid particles which is a prerequisite for DNA packaging (Myers & Carter, 1981). Freeze-thaw lysates of HeLa cells transfected with wild-type or mutated AAV genomes and infected with adenovirus were sedimented by high-speed centrifugation (200000 g for 3 h at 4 °C in a Sorvall TH641 rotor) and the sediments were analysed by Western blotting (Fig. 5b). Sedimentable capsid proteins were
Fig. 5. Analysis of viral ssDNA accumulation and capsid formation. (a) Hirt extracts of HeLa cells transfected with pTAV-2, pTAV-p or pTAV-d, respectively, and infected with adenovirus-2 were prepared and analysed by Southern hybridization. Position of the viral ssDNA (ss), the monomeric ds form (RF1) and the dimeric ds form (RF2) are indicated. (b) Capsid formation was analysed by high-speed centrifugation of freeze-thaw lysates of HeLa cells transfected with wild-type or mutant plasmids followed by adenovirus infection. Particle formation was detected by gel electrophoresis and Western blotting of the corresponding sediments.

detected in lysates of pTAV-2 transfected cells, whereas there was only a minimal amount of sedimented VP3 from lysates of cells transfected with pTAV-p. We failed to detect sedimentable capsid proteins in lysates of cells transfected with pTAV-d, the mutant which was not infectious at all. This result suggests that the mutants pTAV-p and pTAV-d are defective in viral capsid assembly.

Subcellular distribution of the capsid proteins

Transfection of HeLa cells with pTAV-2, pTAV-p, or pTAV-d, respectively, and infection with adenovirus resulted in different subcellular distribution patterns of the capsid proteins as analysed by indirect immunofluorescence. In most cells transfected with pTAV-2 the AAV capsid proteins were equally distributed in nuclei and cytoplasm when probed with a polyclonal antiserum recognizing all three capsid proteins (Fig. 6a). Double immunofluorescence with a monoclonal antibody specific for VP1 and VP2 (see Fig. 4) shows an accumulation of these capsid proteins in the nucleus (Fig. 6a). In a number of cells a weak cytoplasmic staining of VP1 and VP2 could also be observed (not shown). When cells were fixed 24 h after infection with the helper virus, we typically observed a strong nucleolar staining with AAV capsid protein antibodies (Fig. 6a and b). In cells transfected with the mutant plasmids the capsid proteins were detected in the cytoplasm predominantly, often in a granular pattern, sometimes with a preferential localization close to the nuclear membrane (Fig. 6c to f). Formation of such granular VP aggregates seemed to be more prominent in cells transfected with pTAV-d (Fig. 6e and f) than in those transfected with

Fig. 6. Immunofluorescence analysis of wild-type and mutant capsid proteins. The subcellular localization of AAV capsid proteins in HeLa cells transfected with pTAV-2 (a, b), pTAV-p (c, d), or pTAV-d (e, f) and infected with adenovirus-2 was detected by indirect immunofluorescence using a polyclonal VP antiserum (a, c, e) and a monoclonal antibody A69 recognizing VP1 and VP2 only (b, d, e). Note the predominantly cytoplasmic, granular staining of the mutated capsid proteins. Bar marker represents 20 μm.
pTAV-p (Fig. 6c and d). Strikingly, no nucleolar staining was observed in cells transfected with the mutant plasmids at any time point although some capsid protein was detectable in the nucleus.

Discussion

We have re-examined the AAV-VP ORF in the course of the generation of viral capsid mutants which we started to develop based on a predicted RGD integrin-binding motif. DNA sequencing of two independent AAV-2 clones suggested, in addition to several other nucleotide changes, a deletion of C at position 4227 which causes a frameshift and extension of the VP ORF by 27 amino acids. This change in the VP amino acid sequence has been confirmed directly by sequencing of protease-generated peptides isolated from baculovirus-expressed VP3 protein. The DNA used for expression of VP3 was obtained from pTAV-2. The Mø of VP3 expressed in insect cells and derived from pTAV-2 is identical to that of VP3 derived from HeLa cells infected with both AAV-2 strain H (ATCC) and adenovirus (Ruffing et al., 1992). This suggests that the AAV wild-type virus DNA does not contain the C-terminal ORF established by Srivastava et al. (1983), since the Mø difference in the capsid proteins between the baculovirus-expressed and the AAV-derived protein should have been observed. Therefore these peptide sequences demonstrate that the AAV capsid contains no RGD motif which could be involved in binding to integrins and viral uptake.

The two capsid mutants that we generated affect this newly established C-terminal sequence. In the mutant pTAV-d this sequence is deleted in addition to the previously predicted RGD and in pTAV-p I⁴⁰⁰ is changed to S. Both mutants showed no detectable ssDNA accumulation in spite of normal levels of AAV DNA replication. This is probably due to reduced or missing capsid protein oligomerization which correlates with a drastic cellular mislocalization of the mutated capsid proteins in comparison to the wild-type. These mutations are localized in the C terminus of the capsid proteins and affect the ORF of all three capsid proteins. Therefore they cannot be compared with mutations of individual capsid proteins in the N terminus of the capsid proteins (Muralidhar et al., 1994) which also lead to defects in ssDNA accumulation and production of infectious virus.

Our findings suggest that reduced particle formation and ssDNA accumulation of the capsid protein mutants pTAV-p and pTAV-d might be a result of the mislocalization of the mutated proteins. The higher degree of mislocalization of the deletion mutant (pTAV-d; Fig. 6e and f) correlates with the lower amount of particles in the cell lysate and the complete lack of infectivity compared with the mutant pTAV-p. Based on sequence comparisons, the carboxy-terminal mutations should not affect any nuclear localization sequences in the capsid proteins. It has been shown that VP1 and VP2 are able to accumulate in the nucleus (see also Fig. 6b) whereas VP3 equilibrates between nucleus and cytoplasm, consistent with the positioning of nuclear accumulation sequences in the amino terminus of the capsid proteins (Ruffing et al., 1992). The reduced nuclear accumulation of the capsid proteins might thus be explained rather by a conformational change of the capsid proteins that leads to cytoplasmic retention due to interaction with immobilized cytoplasmic components or due to irregular aggregation of the capsid proteins. This explanation is supported by the granular distribution pattern of the mutated capsid proteins in the cytoplasm (Fig. 6c to f) and the lack of soluble but sedimentable capsids (Fig. 5b). Alternatively, the interaction of the capsid proteins with a cytoplasmic component necessary for cotransport into the nucleus, could be disturbed by the C-terminal mutations. Such an interpretation would also explain the loss of nucleolar accumulation of the mutated capsid proteins despite the presence of some capsid protein in the nucleus. In any case, the observed defect in viral assembly correlates with an incomplete nuclear accumulation of the capsid proteins and a lack of transient nucleolar localization. Based on the presented data, it cannot be decided whether the mislocalization of the capsid proteins is a cause or a consequence of the assembly defect. Direct analysis of capsid protein interactions is needed to clarify the biochemical consequences of the introduced capsid protein mutations. It is clear from these data, however, that the corrected C terminus of the capsid proteins, accounting for about 10% of the total capsid amino acid sequence, is important for the generation of infectious virus.

We thank K. Berns for the plasmid pSM620, H. zur Hausen for continuous support and A. Büttner, M. Pawlita and S. Weger for critical reading of the manuscript. We are grateful to U. Ackermann and A. Kern for excellent technical assistance.

References


mammalian cells by plasmid DNA. Molecular and Cellular Biology 7, 2745-2752.


(Received 16 May 1994; Accepted 29 July 1994)