Adenovirus Serotype 30 Fiber Does Not Mediate Transduction via the Coxsackie-Adenovirus Receptor

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Prior work by members of our laboratory and others demonstrated that adenovirus serotype 30 (Ad30), a group D adenovirus, exhibited novel transduction characteristics compared to those of serotype 5 (Ad5, belonging to group C). While some serotype D adenoviruses bind to the coxsackie-adenovirus receptor (CAR), the ability of Ad30 fiber to bind CAR is unknown. We amplified and purified Ad30 and cloned the Ad30 fiber by overlap PCR. Alignment of Ad30 fiber with Ad3, Ad35, Ad5, Ad9, and Ad17 revealed that Ad30, like Ad9 and Ad17, has a shortened fiber sequence relative to that of Ad5. The knob region of fiber was 45% identical to that of the Ad5 knob regions. We made a chimeric recombinant virus (Ad5GFPf30) in which the Ad5 fiber (amino acids [aa] 47 to 582) was replaced with Ad30 fiber sequences (aa 46 to 372), and CAR-mediated viral entry was determined on CAR-expressing Chinese hamster ovary (CHO) cells. While CAR expression significantly increased Ad5GFP-mediated transduction in CHO cells (from 1 to 36%), it did not enhance Ad5GFPf30 gene transfer. Binding of radiolabeled Ad5GFPf30 or Ad30 wild-type virus was also not improved by the expression of CAR. These results suggest that Ad30 fiber is distinct from Ad5, Ad9, and Ad17 fibers in its inability to direct transduction via CAR.

Adenovirus (Ad) tropism is the result of specific binding of the virus to the target cell by means of a cellular receptor. The C-terminal portion of adenovirus fiber, or “knob,” is responsible for the specificity of receptor recognition. The 46-kDa human coxsackie-adenovirus receptor (CAR) interacts with the fiber knobs of representative adenoviruses from most of the defined subgroups (serotypes 2, 4, 5, 9, 12, 15, 17, 19, 31, and 41) (2, 6, 14, 20, 23), indicating that CAR can function as their cellular receptor. The mouse homologue of CAR has also been isolated and shown to mediate infection of human Ad type 2 (Ad2) (3, 18).

CAR is a type I transmembrane protein containing a cytoplasmic COOH terminus and an N-terminal extracellular domain (2, 3, 18). The exoplasmic region contains two immunoglobulin (Ig)-related domains (IgV and IgC2) stabilized by intrachain disulfide bonds and two potential N-linked glycosylation sites. Ad5 fiber interactions with CAR occur within the N-terminal IgV domain (4, 6, 10). Prospective amino acid residues within the fiber required for binding to CAR have been identified (10, 13). By using competitive assays with recombinant fiber, it was found that two amino acids in the AB loop (Ser408 and Pro409), one in the DE loop (Tyr477), and one intrachain disulfide bond (IgV strand F) are necessary for Ad5 binding to CAR (14). We previously showed that another D-serotype virus, Ad30, infected primary endothelial and neuronal cells more efficiently than Ad5 (5). To begin to understand the mechanisms for this difference, we first sought to determine whether Ad30 fiber bound CAR. For these experiments, the Ad30 fiber was first cloned and sequenced. We then tested Ad30 fiber interactions with CAR, using both wild-type Ad30 and a recombinant Ad5 whose native fiber was replaced by that of Ad30.

MATERIALS AND METHODS

Cells and viruses. Human embryonic kidney (HEK 293) cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 1% glutamine, and 1% penicillin-streptomycin. Chinese hamster ovary (CHO) cells were maintained in DMEM F12 medium supplemented with 1% penicillin-streptomycin and 10% FCS. Ad5 (VR-273) was purchased from the American Type Culture Collection and subsequently amplified by infection of HEK 293 cells. Ad30 (VR-273) was purchased from the American Type Culture Collection and subsequently amplified by infection of HEK 293 cells. Viral particles were banded in CsCl gradients, dialyzed, and stored in aliquots at \(-80^\circ\)C. Ad5CMVhCAR (AdhCAR), produced by the University of Iowa Gene Transfer Vector Core, was a kind gift from Joseph Zabner, University of Iowa. Ad5CMVntlacZ (Ad5lacZ), also produced by the University of Iowa Gene Transfer Vector Core, is used routinely in the Davidson laboratory. Sequencing Ad30 fiber protein. Viral DNAs from purified Ad30 particles were isolated by standard protease treatment and ethanol precipitation methods. Primers to the 5' and 3' ends of the fiber gene, designed by means of comparison of the known sequences of four D-serotype viruses, (Ad8, Ad9, Ad15, and Ad7), were used to amplify the fiber gene by PCR. The primers were 5'-CGGGATCCGCACCATGCAAAAGGGAGCTCCGG-3' and the degenerate primer AdDfberR 5'-CGGGATCTTATTCTTGGC...
YTATAAGG-3'), respectively. The fiber gene was completely sequenced in both directions.

**Construction of Ad35GFP30.** The endogenous fiber sequence of Ad5 (nucleotides 31042 to 32787) was replaced with the Ad30 sequence by overlapping PCR. The Ad5 fiber was amplified such that it contained the first 147 bp of the Ad5 tail (bp 31042 to 31189). Overlapping primers specific for the tail-shaft boundary containing 18 bp of Ad5 and 18 bp of Ad30 were generated. In the first phase of the overlapping PCR, two DNA fragments corresponding to the Ad5 tail region and the Ad30 shaft and knob regions were amplified. The tail was generated using primer Ad5fiberforBamH1 (5’-CCGGGATCCGGCAGTAGAAG CGCGCAAGA-3’, bp 31042 to 31189) and 17Adsovertail (5’-GATTGGGGTC GACAGTTGTTAAGAGAGTACCCCAGG-3’) with T7polymerase. The shaft-knob region was amplified with the primers 5Ad17overtail (5’-CTTGGGGTACTCTT GGGGATCCGGCAGTAGAAG-3’) and Ad30RevSpe1 (5’-AAACTAGTGT CATTCTTGGGCTGATA-3’) II. Primers to the 5′ and 3′ ends were designed to incorporate the restriction enzyme recognition sites BamH1 and Spe1, respectively. After 30 PCR cycles, the Ad5 tail and Ad30 shaft-knob region products were purified by agarose gel electrophoresis and then mixed, and the mixture was used as a template with primers Ad5fiberforBamH1 and Ad30RevSpe1 to amplify the entire chimeric 5/30 fiber. The 1.119-kb-long chimeric 5/30 fiber product, containing the Ad5 tail and the Ad30 shaft and knob domains, was purified by agarose gel electrophoresis, digested with NdeI and SpeI, and ligated into pBSR2H1 (22) cut with NdeI and SpeI. The resulting plasmid, pBS-5/30, linearized with NolI and BamH1, and pTGI3602/RSVeGFP/Swa1, based on Xia et al. (22), linearized with SwaI to drive homologous recombination in fiber, were used to cotransform the RecA+ Escherichia coli strain BJ5183. We screened the resulting recombinants by PCR and direct sequencing. Positive recombinants contained the entire Ad5 genome, flanked by pFlu sites with the following modifications: replacement of the E1 region by an expression cassette in which Rous sarcoma virus (RSV)LTR drives expression of enhanced green fluorescent protein (eGFP) and replacement of the endogenous Ad5 fiber with the chimeric 5/30 fiber. This plasmid was then digested with PacI and transfected into HEK 293 cells for the production of viral particles as previously described (1). Cytopathic effect (CPE) was evident 14 days posttransfection in 60-mm2 dishes of HEK 293 cells. Lysates of Ad5RSVeGFPf30 (Ad5GFPf30) were used for further amplification. CPE was evident 40 h postinfection. The virus was harvested and purified by standard methods as described previously (1). The Ad5RSVeGFP control virus (Ad5GFP), with nonrecombinant fiber, was similarly generated. Both viruses were analyzed by plaque assay on HEK 293 or A549 cells. The amino acid sequence of Ad30 is shown in Fig. 1. At the protein level, Ad30 fiber (10, 13). Of the four residues shown to be important in CAR binding (9, 10, 13), only one (Arg 481) was conserved in Ad30 fibers. As sequence data were acquired, further specific primers were designed and employed until the entire sequence of the Ad30 fiber gene was obtained. The amino acid sequence of Ad30 is shown in Fig. 1. Ad30 fiber is very similar to other known D-serotype fibers, namely, Ad9 and Ad17, and less so to Ad3 or Ad35 fiber. We found the most divergence between Ad30 and Ad5 fibers.

At the protein level, Ad30 fiber shares 25% overall identity with Ad5 fiber. When analyzed according to regions within the fiber protein, 61, 11, and 45% identities were found in the tail, shaft, and knob regions, respectively. The 11% identity in the shaft region resulted primarily from differences in the shaft lengths of the two fibers. Ad30 fiber is 209 amino acids shorter than Ad5. In contrast, 98, 55, and 66% identities were found between the Ad30 and Ad9 tail, shaft, and knob regions, respectively. Ad30 fiber was 66% identical to the Ad9 fiber.

Recent work investigated the binding of recombinant fibers to immobilized CAR and found specific residues important in CAR binding (9, 10, 13). Of the four residues shown to be critical for CAR binding (Ser490, Pro490, Tyr577, and Leu485), all but one (Leu485) were conserved in Ad30 fiber (10, 13). Of the three amino acid residues which may peripherally affect CAR binding, (Ala406, Arg412, and Arg481), only one (Arg412) has been conserved. These similarities and differences are illustrated in Fig. 1.
FIG. 1. Comparison of the amino acid sequences of adenoviral fibers from the subgroup D Ads (serotypes 30, 9, and 17) with those from subgroup C (serotype 5) and subgroup B (serotypes 35 and 3). The amino acid positions are based on those of Ad5. Blocked letters indicate amino acids found to be important for CAR binding (residues at positions 406, 408, 409, 412, 417, 477, 481, and 485, based on the Ad5 protein sequence).

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Tail

D 30 MSKLKRD, AD DNQPYYQGQ ARN, QNIPL TPQVSDGQ, #NPQPPV
C 5 M, KRASPPED TPQPYYQT, TQGSSPFLQ, #QSSPPP
D 9 MSKLKRD, AD DNQPYYQGQ ARN, QNIPL TPQVSDGQ, #NPQPPV
D 17 MSKLKRD, AD DNQPYYQGQ ARN, QNIPL TPQVSDGQ, #NPQPPV
B 35 MTQRRS, SD SLPQPYYQGQ ARN, QNIPL TPQVSDGQ, #NPQPPV
B 3 MAKRLA, ST SLPQPYYQGQ ARN, QNIPL TPQVSDGQ, #NPQPPV

Shaft

LSLKLADP, SSA TIPQGSDGQ, #NPQPPV
LSLKLADP, SSA TIPQGSDGQ, #NPQPPV
LSLKLADP, SSA TIPQGSDGQ, #NPQPPV
LSLKLADP, SSA TIPQGSDGQ, #NPQPPV
LSLKLADP, SSA TIPQGSDGQ, #NPQPPV

Knob

30 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
35 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
36 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
37 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
38 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
39 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
40 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
41 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
42 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
43 YN...PVPIK ITPFNEAD...#SAYSI TIPYSWTDQ. YDNPFPDDSTTEPSTIQAQ
Chimeric fiber. To test the properties of the Ad30 fiber independent of the Ad30 virion, a chimeric virus (Ad5GFPf30) was generated by homologous recombination in E. coli. (1). The Ad30 fiber gene was generated by overlapping PCR and, similar to earlier work with Ad17 fiber (5), was designed to retain the Ad5 tail and replace Ad5 shaft and knob sequences with those from Ad30. The fiber fragment was cloned into the Ad5 backbone in place of the endogenous Ad5 fiber as depicted in Fig. 2. Sequence analysis of plasmid pAd5GFPf30 confirmed the presence of the chimeric fiber. pAd5GFPf30 was linearized by PacI and transfected into HEK 293 cells to generate virus (1). Western blot analysis of purified particles verified the expression of the shortened chimeric fiber (Fig. 3). Ad5GFPf30-induced CPE was delayed relative to that of Ad5GFP (45 versus 30 h), and viral yields (numbers of particles and infectious units) were lower by two- to threefold. The titer of Ad5GFPf30 was $4 \times 10^9$ PFU/ml, and that for Ad5GFP was $1 \times 10^{10}$ PFU/ml.

Ad5 has been shown to infect cells via CAR (2, 6, 14, 20, 23). To assess the potential use of CAR by the hybrid virus, CHO cells were used. This cell type has been shown to express little if any endogenous receptor (16, 18) but, when made to express CAR, can direct Ad5-mediated gene transfer. Ad5GFP infected 1% of control-virus-transfected CHO cells (Fig. 4), confirming previously published data (2, 18). Similar results were seen with Ad5GFPf30 (Fig. 4). To convert to a CAR-expressing phenotype, CHO cells were incubated with an Ad5hCAR-CaPi coprecipitant for 30 min at 37°C, resulting in 96% of cells
We cloned, sequenced, and analyzed Ad30 fiber for its ability to bind CAR. A direct amino acid sequence comparison of Ad30 fiber with that from Ad5 demonstrated disparate as well as highly homologous regions. Sequences within the tail were most conserved, followed by the knob region. Within the knob, there was surprising conservation of amino acid sequences, and in the region of the knob shown to be important in CAR binding by competitive or cell-free surface plasmon resonance assays (10, 13), there were homologous or conservative amino acid substitutions in seven of nine instances. We presumed that assays (10, 13), there were homologous or conservative amino binding by competitive or cell-free surface plasmon resonance in the region of the knob shown to be important in CAR most conserved, followed by the knob region. Within the knob, residues Ser408, Pro409, Lys417, Lys420, Tyr477, and Arg481, shown to be important in CAR binding, were found to be identical between Ad5 and Ad30. Mutations at Ala406, Arg412, and Leu485 of Ad5 diminish CAR binding (10). At these sites, Ad30 differs. However, these substitutions (Asp406, Lys412, and Asp485) are also present in Ad9 and/or Ad17, indicating that these changes alone are probably not sufficient for inhibiting binding to CAR.

More distinctive between Ad30 and the CAR-binding viruses are the amino acids surrounding these critical regions. The Ala406 in Ad5 or the Asp406 in Ad30, Ad9, and Ad17 is an example. In Ad5, Ad9, and Ad17, there is an invariable proline at amino acid position 405 and a serine or threonine at position 407. Ad30 possesses a leucine and proline at positions 405 and 407, respectively. And although Tyr477 is conserved in Ad30, Ad5, Ad9, and Ad17, the residues flanking Tyr477 are distinct when Ad30 is compared to the CAR-binding fibers.

The Ad30 fiber shaft, similar to those of other D-serotype viruses, was found to be short relative to the Ad5 shaft. Nonetheless, we found significant homology for the first 70 amino acids, as well as for regions near the hinge regions, notably from residues at positions 378 to 400. Serotype B viruses Ad5 and Ad3 also have short shaft lengths relative to that of Ad5. A study by Shayakhmetov and Lieber suggests that shaft length is important in CAR binding for Ad5 and Ad9; when Ad5 or Ad9 knobs were placed onto shorter shafts, CAR interactions were impaired (15). However, fiber length was not critical for Ads entering cells via a non-CAR-dependent pathway, as was evident for Ad35 (15). The rationale for this difference is suggested to be a charge repulsion between the negatively charged region within hypervariable region 1 of Ad5 hexon and acidic proteins on the cell surface. The hexon sequence of Ad30 is not presently available. As such, it would be interesting to test if the Ad30 knob, placed onto a longer shaft, would ameliorate the inability of Ad5GFPf30 to bind CAR.

Ad30 wild-type and the chimeric AdGFPf30 viruses were propagated in HEK 293 cells at levels similar to each other but less efficiently than were Ad5 fiber-expressing viruses. It is possible that Ad30, like Ad9, is more dependent on penton base interactions with cell surface integrins for viral entry (10, 12), although the presence of RGD motifs in the Ad30 penton base is currently unknown. Similarly, the shorter shaft of the Ad30 fiber may allow for more appropriate contact between the Ad5 penton base on Ad5GFPf30 capsids and cell surface α₅-integrins (12).

In summary, we sequenced and analyzed the fiber from Ad30, a D-serotype virus, and found it to possess characteristics different from those of previously reported D-serotype virus fibers. It does not bind CAR, although it retains many of the sequences within the knob region predicted to be involved in CAR binding. We speculate that the Ad30 fiber may be advantageous for directing gene transfer to non- or low-level-CAR-expressing cells.

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