

# Modification of the Ad35 fiber to ablate CD46 binding

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## INTRODUCTION

- Neutralizing antibodies to Ad5 in serum can be detected quite readily within various human populations, while the prevalence of antibodies that neutralize Ad35 is much lower. From this perspective Ad35-based vectors may provide an attractive alternative to Ad5-based vectors, although the impact of these antibodies may differ with the application and route of administration.
- Binding of the fiber protein of Ad35 to CD46 could enhance transduction of target tissues that express little or no CAR (Ad5 receptor), e.g. tumor, hematopoietic, and dendritic cells. On the other hand, stimulation of CD46 signalling may have a negative impact.
- We wanted to develop an Ad35 vector that did not bind CD46 in order to evaluate the effects of receptor binding on vector performance. We took the approach of ablating CD46 interaction which could subsequently be adapted for incorporation of novel receptor specificity.

## Subgroup B fibers and CD46 binding

- Most adenoviruses of subgroup B, of which Ad35 is a member, utilize CD46 as a fiber attachment receptor. Ad3 and Ad7 appear to be exceptions among this subgroup in not binding to CD46.
- We compared the sequences of published subgroup B fiber knobs. The sequences fall into four clusters which do not correlate with whether they bind to CD46 or not:

Ad35, Ad21, Ad34, Ad50	92-99% identical
Ad7*, Ad11, Ad14, Ad34a	90-93% identical, 36-38% different from Ad35
Ad16	Differs from both Ad35 and Ad11 by 37%
Ad3*	Differs from groups 1 and 2 by 34-39% and from Ad16 by 25%

\*does not bind CD46

- Adenovirus fibers exhibit a conserved 8-stranded anti-parallel beta barrel at the core of each subunit of the trimer. The individual strands are designated A through J.

## Ad35/5 Knob loop exchanges

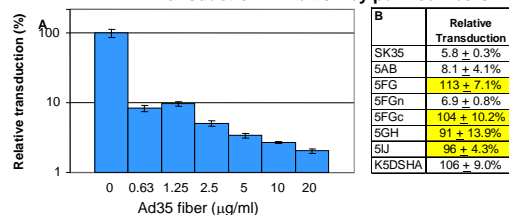
Mutation	Expression
5AB	+
5CD	-
5DE	-
5FG	+
5FGn	+
5FGc	+
5GH	+
5HI	-
5IJ	+

**Fig.1** An alignment of the amino acid sequences of Ad35, Ad5 and Ad3 was constructed. The crystal structures of Ad3 and Ad5 were referenced for identifying the individual loops in the fibers and for identifying the putative loops in the Ad35 knob. The Ad35 sequence was replaced with the corresponding loop from Ad5. In the case of 5AB we used a modification of the Ad5 sequence which disrupted CAR binding (S408E, A415G-E416G-K417G). The FG loop was also divided into two halves at the conserved Phe-Met-Pro motif and exchanged separately (5FGn and 5FGc, respectively).

The knobs were expressed from baculovirus as part of full length fibers containing the tail region of Ad5 fiber and the shaft of Ad35. An N-terminally inserted 6-His tag was used for detection and purification. Those proteins marked with a + for expression were readily recovered as soluble proteins. The 5CD, 5DE and 5HI proteins were recovered at lower levels and were not analyzed for function.

- Four of the seven complete loop exchanges resulted in stably expressed, soluble protein.

## Transduction inhibition by purified fibers

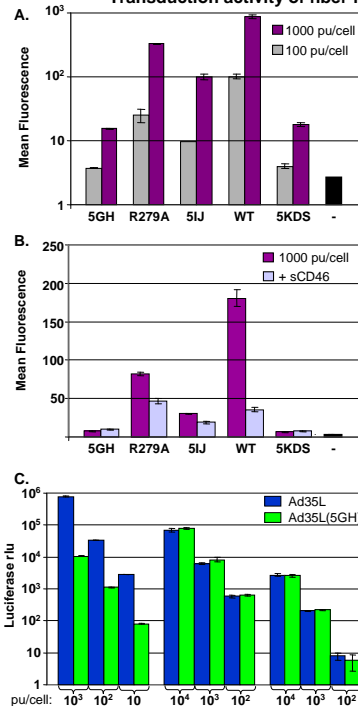


**Fig. 2 A.** Transduction of 293 cells by Ad35L, an E1-deleted Ad35 vector expressing luciferase, is blocked by baculovirus-expressed Ad35 fiber. Purified fiber was quantitated by BCA assay and added to adherent cells at the indicated concentrations. After 1 hr, Ad35L was added (100 pu/cell) and incubated with cells in the presence of fiber for an additional hour. Cells were harvested at 18 hr to assay for luciferase and transduction was normalized to that in the absence of fiber (100%).

**B.** Purified Ad35 fibers carrying loop exchanges were analyzed for their effect on transduction when included at 5 µg/ml in the assay described in A. The K5DSHA fiber carries the Ad35 fiber shaft and a CAR-binding ablated Ad5 knob.

- The 5FG, 5GH, and 5IJ loop exchanges lost the ability to block transduction.
- Analysis of 5FGn and 5FGc indicated that it is the C-terminal half of the loop that has an effect on transduction.

## Transduction activity of fiber-modified vectors



**Fig. 4.** Mutant fibers selected on the basis of their reduced binding to CD46 were incorporated into E1-deleted Ad35 vectors expressing GFP. Plasmids carrying the recombinant Ad genomes were transfected into 293-ORF6 cells and lysates were passaged until vector grew out. Vectors were produced in suspension 293-ORF6 cells and purified by three successive cesium gradients.

**A.** To assay transduction of the purified vectors, suspension 293-ORF6 cells were inoculated with vector at the indicated pu/cell and assayed by FACs after 20 hr. 5KDS is an Ad35 vector with a CAR binding-ablated, Ad5 fiber knob.

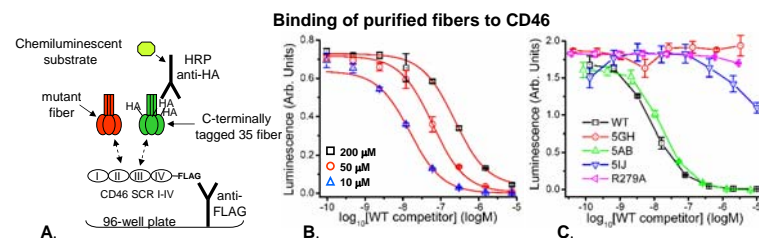
- The 5GH mutant vector exhibited the lowest transduction activity, behaving similarly to K5DS.

**B.** Transduction inhibition by soluble CD46 (added at 5 µg/ml).

- The R279A and 5IJ mutants, which gave reduced transduction relative to WT, still showed some blocking by CD46. No effect was seen for CD46 on the low transduction by 5GH and 5KDS.

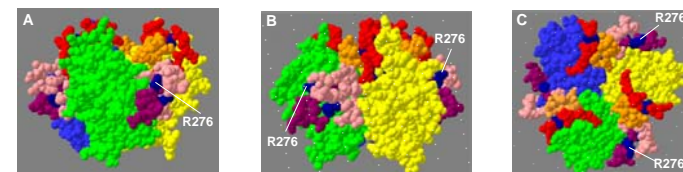
**C.** The 5GH mutation was built into an E1-deleted, luciferase expressing Ad35 vector, Ad35L(5GH). Transduction by Ad35L(5GH) was measured in comparison to Ad35L, the latter differing only in having the wild-type fiber.

- Transduction of CD46-positive 293 cells by Ad35L(5GH) was reduced by 1.5-2 logs relative to Ad35L. This reduction is comparable to that seen in the fiber-blocking experiments.
- By contrast, the two vectors gave similar transduction on CD46-negative CHO and B16F10 cells.



**Fig. 3 A.** Assay for binding of purified fibers to CD46. A FLAG epitope and termination codon were inserted immediately following the fourth short consensus repeat (SCR) in the extracellular domain of CD46 for expression using baculovirus. White, 96-well plates were coated with anti-FLAG antibody and then washed and blocked (BSA) before adding soluble CD46. Mixtures of C-terminally HA-tagged Ad35 fiber with untagged wt (B) or mutant Ad35 fibers (C) were prepared and added to wells to allow binding to CD46. Bound HA-tagged fiber was detected with an anti-HA antibody and chemiluminescence.

- Global fitting directly to the 3 binding curves in B, indicates that the Kd for wt Ad35 is 4.0 ± 0.7 nM.
- The 5GH loop exchange showed no detectable binding to sCD46, while 5IJ showed reduced binding. In contrast, 5AB gave a binding curve similar to wt (Kd = 7.1 nM).
- The R279A point mutant in the HI loop showed no detectable binding to sCD46.



**Fig. 5.** Space-filling display of Ad3 knob amino acid residues, colored by chain (A, B, C) or loop (FGc, GH, HI, IJ) [PDF 1HTZ displayed with SwissPdb Viewer (<http://www.expasy.org/spdbv/>)]. The HI loop amino acid R276, which is the equivalent of the Ad35 residue changed in R279A, is also highlighted. Panel A shows the knob from the side with chain c in the front, while the knob in B has been rotated roughly 60° so that the junction between chains c and a is at the front. Panel C shows the knob from the top.

- This display shows how the FGc loop and neighboring HI loop of one monomer abuts the GH loop and neighboring IJ loop of an adjacent monomer. This extended surface of the knob contains the mutations we have identified that impact CD46 binding.
- Although Ad3 differs from Ad35 fiber in not binding to CD46, the structure of the Ad11 knob, in complex with a fragment of CD46, has recently become available [Persson *et al.*, *Nat. Struct. Mol. Biol.* 14:164]. That structure indicates contact between CD46 and the DG, HI and IJ loops of Ad11, where the IJ loop is part of a second knob protomer.

	FGc	GH	H	IJ
Ad35	S T T A Y P F N T T R D S	M T S Y D R S L	S R M I S S N V A	S E S P E - - S N I A
Ad11	S T T A Y P F N D N S R E K	T A S - D R T A	R R A L N D E T S	G D A P E V Q T S A T
Ad5	S T T A Y P F L P N A G T H N	K A S - D G A L	K R L P D S R T S	G L A P E - - T T Q A
Ad5	N L S A Y P K S H G K T A K	L N G - D K T K	G T Q E T G D T T P S A	S G H - N - - Y I N E

**Fig. 6.** Alignment of amino acid sequences in the indicated loops for Ad35, Ad11, Ad3 and Ad5. The residues underlined in the Ad11 sequence were reported to make close contact with CD46.

- The sequence diversity between Ad35 and Ad11 suggests that while similar regions of these fibers may interact with CD46, the individual residues that mediate binding may differ.

## SUMMARY

- We have found that the Ad35 fiber knob was receptive to loop exchanges with Ad5 in the AB, FG, GH, and IJ loops. In the case of the large FG loop we were also able to generate functional fibers by replacement of either the N- or C-terminal half of the loop.
- 3 of the 4 loop exchanges affected the ability of the fiber to bind to CD46. Involvement of multiple loops in this binding is consistent with a structural model based on Ad3 as well as recent data on the Ad11 knob.
- Site-directed mutagenesis analysis which yielded the R279A mutant indicated that the HI loop may also contribute to CD46 binding.
- The 5GH loop exchange in the Ad35 fiber had the most extensive effect on CD46 binding, both in the protein-binding assay and in the context of vector transduction.
- Ad35 vectors that do not bind CD46 will be useful tools for exploring the effect of receptor interaction on vector performance and for developing vectors that are engineered with novel receptor specificity.