Adenovirus type 5 fiber knob domain has a critical role in fiber protein synthesis and encapsidation

Petra Henning,1,2 Emma Lundgren,1 Mattias Carlsson,1 Karolin Frykholm,1 Jenny Johannisson,1 Maria K. Magnusson,1,2 Erika Täng,1 Laure Franqueville,3 Saw See Hong,3 Leif Lindholm1,2 and Pierre Boulanger3,4

Correspondence
Leif Lindholm
leif.lindholm@gotagene.se
Pierre Boulanger
Pierre.Boulanger@sante.univ-lyon1.fr
1Got-A-Gene AB, Östra Kyviksvägen 18, SE 42930 Kullavik, Sweden
2Institute for Biomedicine, Department of Microbiology and Immunology, University of Göteborg, PO Box 435, SE 40530 Göteborg, Sweden
3Laboratoire de Virologie et Pathogénèse Virale, Université Claude Bernard de Lyon and CNRS UMR-5537, Faculté de Médecine RTH Laennec, 7 Rue Guillaume Paradin, 69372 Lyon Cedex 08, France
4Laboratoire de Virologie Médicale, Domaine Rockefeller, Hospices Civils de Lyon, 8 Avenue Rockefeller, 69373 Lyon Cedex 08, France

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Adenovirus serotype 5 (Ad5) vectors carrying knobless fibers designed to remove their natural tropism were found to have a lower fiber content than recombinant Ad5 with wild-type (WT) capsid, implying a role for the knob-coding sequence or/and the knob domain in fiber encapsidation. Experimental data using a variety of fiber gene constructs showed that the defect did not occur at the fiber mRNA level, but at the protein level. Knobless fiber proteins were found to be synthesized at a significant slower rate compared with knob-carrying fibers, and the trimerization process of knobless fibers paralleled their slow rate of synthesis. A recombinant Ad5 diploid for the fiber gene (referred to as Ad5/R7-ZZwt/E1 : WT-fiber) was constructed to analyse the possible rescue of the knobless low-fiber-content phenotype by co-expression of WT fiber. Ad5/R7-ZZwt/E1 : WT-fiber contained a knobless fiber gene in its natural location (L5) in the viral genome and an additional WT fiber gene in an ectopic position in E1. Knobless fiber was still synthesized at low levels compared with the co-expressed E1 : WT fiber and the recovery of the two fiber species in virus progeny reflected their respective amounts in the infected cells. Our results suggested that deletion of the fiber knob domain had a negative effect on the translation of the fiber mRNA and on the intracellular concentration of fiber protein. They also suggested that the knob control of fiber protein synthesis and encapsidation occurred as a cis effect, which was not modified by WT fiber protein provided in trans by the same Ad5 genome.

INTRODUCTION

One of the possible strategies for redirecting adenovirus serotype 5 (Ad5) vectors to desired cell targets consists of (i) removing their natural tropism and (ii) inserting a new cell-binding ligand in one of the capsid proteins. Step (i) can be achieved by deletion of the entire fiber knob domain and its replacement by a non-viral trimerization motif. In earlier studies, we and others have described the construction of viable, knobless adenoviruses in which the deletion of the knob was compensated for by the incorporation of sequences encoding a non-viral trimerization signal (Krasnykh et al., 2001; Magnusson et al., 2001; van Beusechem et al., 2000). However, such recombinant viruses showed some defective functions compared with wild-type (WT) virus: growth and infectivity were impaired, virus entry was hampered in some cases and the fiber content of virus particles carrying knobless fibers was lower than the theoretical number of 12 copies per virion. In contrast, recombinant Ad5 with fibers carrying their knob domain in addition to new cell ligands have nearly normal fiber numbers, suggesting that the knob domain plays a role, direct or indirect, in the fiber content of the virion (Henning et al., 2002, 2005; Magnusson et al., 2001, 2002; van Beusechem et al., 2000).

The lower growth rate and infectivity index of our Ad5 vectors could be due to negative effects on multiple steps of the virus life cycle, including their binding to cellular receptors, endocytosis, intracellular trafficking, assembly,
maturation and cellular release. Ad5 normally infects cells by binding to the high-affinity cellular receptor CAR (coxsackievirus–adenovirus receptor) via the knob domain of the fiber, followed by internalization mediated by cell-surface αvβ3/5 integrins and RGD motifs in the penton base (reviewed by Russell, 2000). In the early steps of virus infection, the length and flexibility of the fiber have been shown to be of importance for efficient virus entry, at least when the cellular receptor used by the virus is CAR (Shayakhmetov & Lieber, 2000; Wu et al., 2003). Thus, shortening of the fiber shaft down to seven shaft repeats (R7), which is the norm in most of our constructs, could negatively affect cell entry of the virus as a consequence of the modified fiber shaft flexibility. At late stages of the virus cycle, the fiber and its knob domain have been shown to carry out other important functions, such as virus maturation, intracellular trafficking and membrane lysis (Gaden et al., 2004; Legrand et al., 1999; Leissner et al., 2001; Miyazawa et al., 1999, 2001; Shayakhmetov et al., 2003; von Seggern et al., 1999).

The fiber polypeptide is a late product and is encoded in transcription unit L5 of the adenovirus genome (Shenk, 2001). L5 is the last of five cassettes of transcripts (L1–L5) in a large mRNA transcript with a tripartite leader sequence, processed into five individual mRNA families via alternative splicing and usage of different poly(A) signals. Expression of the late mRNA families is controlled by the major late promoter, which becomes increasingly active at late stages of infection (Shaw & Ziff, 1980). Translation of the five late mRNA families seems to be dependent on the 5’-tripartite non-coding region present in all of the families (Berkner & Sharp, 1985). To our knowledge, no separate regulation of translation of the fiber mRNA has been assessed. The only gene encoding structural elements of the virion that was changed in our recombinant Ad5 genomes was the fiber gene, which was justified by the modification of cell tropism.

Efforts to increase the fiber content of retargeted Ad5 is the first step in the improvement of vector infectivity, as it is essential that viruses used for gene therapy have optimal biological activity, in particular to minimize the number of virus particles that have to be administered. One of the possible strategies is to maintain the fiber knob domain in the fiber construct, but to introduce a restriction protease site upstream of the knob and downstream of the retargeting cell ligand. It is then possible to grow stocks of knob-carrying Ad5 vectors to high infectious titers and to obtain knobless fiber adenovirus vector following proteolytic removal of the knob (Hong et al., 2003).

In order to find alternative strategies of correction, we investigated the molecular mechanisms responsible for the lower fiber copy numbers of knobless recombinant Ad5. The expression of knobless fiber genes in adenovirus-infected cells was analysed at different levels: transcriptional, post-transcriptional, translational and post-translational. The only difference between knob-carrying and knobless fibers was the slower rate of protein synthesis for the latter, with a concomitant lag in the occurrence of knobless fiber trimer, suggesting that the defect in knobless fiber expression resides at the translational step of the fiber mRNA. The relatively low intracellular content of fiber protein paralleled the low level of incorporation of knobless fibers into virus capsid. This observation has important implications in terms of adenovirus vector technology and strategies for cell targeting via ligand insertion into capsid proteins.

**METHODS**

**Correction of the poly(A) signal in fiber genes.** In our original pGAG3 vector used to clone recombinant Ad5 fibers (Magnusson et al., 2001), the poly(A) signal was altered from AATAAA to AATAAC due to the incorporation of a Xhol cloning site. A new pGAG3 vector was constructed by inserting an adapter from the Xhol site to the Muni site downstream of the fiber gene in the Ad5 genome, generating the sequence TAACTCGAGGCCCAAGAATTAAC. Fibers cloned into this vector had a Xhol site (shown in bold) directly after the fiber stop codon, followed by the last three amino acids of the knob, the poly(A) signal (shown in bold italics) and thereafter the WT untranslated fiber sequence. The R7-ZZwt fiber (Henning et al., 2002; Magnusson et al., 2001, 2002) was cloned in the new pGAG3 vector and rescued into the Ad5 genome and designated R7-ZZwt/corr-poly(A). Next, the WT fiber gene was amplified from the 20th shaft repeat to the Muni site after the fiber using PCR primers 5’-GGGCTCGAGTCAAAAAAATTGGCCCAT (5’ primer, 20th repeat) and 5’-GGGAATTCATAAACACGTGGAA-3’ (3’ primer, Muni site). The 5’ primer added a Xhol site, allowing ligation downstream of recombinant fibers in pGAG3 using Xhol/MunI digestion. R7-ZZwt/r20-Cter was constructed by inserting the sequence from repeat 20 to the knob after the stop codon of the R7-ZZwt fiber and was rescued into the Ad5 genome.

**Reinsertion of fiber knob or shaft sequences in fiber genes (Fig. 1).** Fiber R7-knob was digested with BstXI, which cleaves the Ad5 fiber gene sequence between codons 477 and 478. An adapter was then inserted to allow ligation of the ZZwt tandem ligand, giving rise to R7-401–477-ZZwt. Two different fibers with additional portions of the knob domain inserted downstream of the ZZwt ligand in the R7-ZZwt backbone were constructed and called R7-ZZwt-572–581, respectively. TLWT corresponded to the peptide motif of the shaft–knob junction and peptide 572–581 to the last 10 residues of the knob domain. Fibers with longer shafts were also constructed: they contained repeats 1–10 (R10) and 1–13 (R13), respectively. Their coding sequences were amplified by PCR using the primers 5’-GAAAATAGCCCTGCAAACA (20th primer) and 5’-TTTCCAGGCTAGCTTGCCTTGTGAATCA (3’ primer, 10th repeat) or 5’-GGGCTCGAGTCAAAAAAATTGGCCCAT (5’ primer, 20th repeat) and 5’-GGGAATTCATAAACACGTGGAA-3’ (3’ primer, Muni site). The 5’ primer added a Xhol site, allowing ligation downstream of recombinant fibers in pGAG3 using Xhol/MunI digestion. R7-ZZwt/r20-Cter was constructed by inserting the sequence from repeat 20 to the knob after the stop codon of the R7-ZZwt fiber and was rescued into the Ad5 genome.

**Ectopic insertion of the WT fiber gene.** The WT fiber gene was inserted into E1 in the cloning plasmid pAdTrack-CMV (He et al., 1998) and recombinated with pAdEasy-1. This generated an Ad5 genome in which the E1 region was replaced by the WT fiber gene driven by the intermediate-early cytomegalovirus (CMV) promoter and the green fluorescent protein (GFP) gene driven by a second CMV promoter. This fiber-diploid genome, designated Ad5/WT-fiber/E1:WT-fiber, was then restricted with PacI/SpeI and cosmids cloned with the R7-ZZwt fiber, as described previously (Magnusson et al., 2001), generating Ad5/R7-ZZwt/E1:WT-fiber.
**Production of recombinant adenovirus.** Recombinant fibers were rescued into the L5 region of the Ad5 genome in which the E1 region was replaced by the GFP gene (Magnusson et al., 2001). Knobless Zwt-ligated adenovirus was grown in 293 cells. Other recombinant adenoviruses were grown in 293 cells. Virus titres, expressed as p.f.u. ml⁻¹, were determined by end-point dilution (O’Reilly et al., 1994). The number of physical particles in virus samples was determined by optical absorbance (Mittereder et al., 1996) or by penton base protein assay on immunoblots or Coomassie blue-stained gels (Hong et al., 2003).

**Antibodies and immunological analyses.** The anti-fiber mAbs 4D2.5 and 2A6.36 (Hong & Engler, 1991) were obtained from Jeff Engler (University of Alabama at Birmingham, AL, USA) and mAb RL2, specific for peptide O-linked N-acetylgalcosamine (GlcNAc) residues, from Larry Gerace (Scripps Research Institute, CA, USA). Antibody against fiber (laboratory-made) was raised in rabbit against recombinant adeno-viruses was grown in 293 cells. Virus titres, expressed as p.f.u. ml⁻¹, were determined by end-point dilution (O’Reilly et al., 1994). The number of physical particles in virus samples was determined by optical absorbance (Mittereder et al., 1996) or by penton base protein assay on immunoblots or Coomassie blue-stained gels (Hong et al., 2003).

**Ribonuclease protection assays (RPAs).** Fiber RNA content in adenovirus-infected cells was measured using an RPA (RPA III; Ambion). Hexon RNA was used as an internal standard. Hexon probe was generated by PCR amplification of a 225 bp hexon gene fragment. The fiber probe was PCR amplified with primers generating a 474 bp fragment of the fiber gene 5' sequence common to both the WT and the recombinant fibers. Both probes were cloned into pCR2.1 vectors in both directions to get both sense and antisense strands (original TA Cloning kit; Invitrogen). The antisense strand was used for probing RNA synthesis, while the sense strand was used as the control. Probes were synthesized according to the Maxiscript manual (Ambion), by adding [³²P]UTP (10 mCi ml⁻¹, 50 µCi per probe; Amersham Biosciences). RNA was extracted from adenovirus-infected 293Fc3(1) cells using an RNeasy mini kit (Qiagen) at 24 and 30 h p.i. and RPAs were performed according to the manufacturer’s instructions. In each sample, 2 µg RNA was mixed with fiber and hexon probes (60,000 c.p.m. each) and hybridized at 42 °C overnight. Samples were treated with RNase for 30 min, precipitated, denatured and analysed by PAGE. After transfer to Whatman filters, the amount of radioactivity in the fiber and hexon RNA bands was determined using a Phospholmager.

**Real-time PCR.** RNA was extracted from Ad5/WT-fiber-, Ad5/WT-fiber/Xhol- and Ad5/R7-ZZwt-infected 293Fc3(1) cells at 36 h.p.i. (5 p.f.u. per cell), as described for the RPA. RNA samples, in triplicate, were cleared of possible DNA contamination using a DNA-free kit (Ambion). Cleared RNA was converted to cDNA using the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) with oligo(dT) primers. cDNA samples were subsequently analysed in a comparative and quantitative real-time PCR, using specific primers for both target (fiber) and endogenous control (hexon) genes, SYBR Green PCR master mix (Applied Biosystems) and the ABI Prism 7900 Sequence Detection System (Applied Biosystems, also referred (Hyperfilm MP; Amersham Biosciences). Protein bands were excited from blots and radioactivity was measured in a Beckman LS-6500 scintillation counter (Huvet et al., 1998). Alternatively, bands on autoradiograms were quantified using the VersaDoc Imaging system (Bio-Rad) with the QUANTITY ONE program. Quantification of fiber trimers was performed using electrophoresis of non-denatured proteins in SDS-containing gels, a method termed NDS-PAGE (Magnusson et al., 2001, 2002; Novelli & Boulanger, 1991a).

**Pulse–chase labelling.** 293Fc3(1) cells were infected with adenovirus (10 p.f.u. per cell) for 2 h in six-well plates. Cells were then incubated in complete medium for 19 h, followed by incubation in methionine-free medium (Gibco) for 30 min and pulse-labelled for 5 min with [³⁵S]methionine [15 mCi (555 MBq) ml⁻¹, 400 µCi (14.8 MBq) per well; Amersham Pharmacia]. Cells were harvested immediately after the pulse in 500 µl 10 mM Tris/HCl (pH 8), 10 mM EDTA, 2 × protease inhibitor (Complete EDTA-free protease inhibitor cocktail tablets; Roche). For pulse–chase labelling experiments, adenovirus-infected 293Fc3(1) cells were pulse-labelled for 45 min with [³⁵S]methionine [180 µCi (6.6 MBq) per well at 21 h post-infection (p.i.)] and the radioactivity was chased by incubating cells with 2 mM cold methionine in Iscove’s medium for 2 min (time point 0), followed by further incubation in 1:5 ml Iscove’s medium for 3, 6 and 9 h. Viral proteins were analysed simultaneously by SDS-PAGE in two separate gels, one dried and exposed to X-ray film and the other blotted to a PVDF membrane. Fiber and penton base proteins were located on the autoradiogram by comparison with the immunoblot. Quantification of the fiber and penton base was performed using a Phospholmager (Molecular Dynamics) and IMAGEQUANT software. Fiber protein amounts were corrected and normalized by taking into account the number of methionine (Met) residues in each fiber species and the 12 Met residues in the penton base. Results were expressed as fiber:penton base ratios.
to as TaqMan). The primer pairs used were 5’-CTCCAACTGTGCC-TTTTC-3’ and 5’-GGCTCACAGTTGTTACATT-3’ for the fiber and
5’-GTCCTACCTTGTTGTGTC-3’ and 5’-TGCTTCCCCAGTA-CITGG-3’
for the hexon. The relative amount of RNA was calculated using the formula 2^−ΔCt for the amount of fiber RNA normalized
to hexon RNA, where Ct is the threshold cycle and ΔCt is the
target gene (fiber) minus Ct of the reference gene (hexon).

RESULTS

Nomenclature

The low-fiber-content phenotype observed for knobless adenovirus virions theoretically could be due to multiple mechanisms occurring at the RNA and protein levels and these were explored using a panel of adenovirus vectors with different fiber gene constructs, schematically represented in
Fig. 1 and Table 1. Our prototype vector (abbreviated to Ad5/name of recombinant fiber) contained the fiber gene sequence encoding the fiber tail and the N-terminal third of the shaft domain, the coding sequence for a non-viral trimerization peptide from lung surfactant protein D (neck region peptide; NRP), an oligopeptide spacer (SpA) and a cell-binding ligand, upstream of the knob or in lieu of the deleted knob (Magnusson et al., 2001, 2002). Recombinant fibers were designated R7, R10 or R13, standing for 7, 10 and 13 shaft repeats, respectively, followed by the name of the new cell ligand (RGD or Zwt) with or without the suffix knob. RGD is the tripeptide motif recognized by αvβ3/5 integrins involved in adenovirus endocytosis (Russell, 2000). Zwt, inserted as a single or tandem (ZZwt) ligand, is an IgG-binding domain of 58 residues derived from Staphylococcus aureus protein A, which adopts a three-helix-bundle conformation and is the leader molecule of the Affibody family (Henning et al., 2002, 2005; Nygren & Skerra, 2004). In some cases, a factor Xa cleavage site was inserted at the ligand–knob junction to remove the knob domain proteolytically as desired (Hong et al., 2003).

Influence of the fiber mRNA poly(A) signal and untranslated 3’ sequence on the low-fiber-content phenotype

In our original Ad5 vectors, the fiber mRNA poly(A) signal was changed from AATAAA to ACTAAC for the RGD-ligated fibers and to AATAAC for the Zwt-ligated fibers (Table 1). The possibility that these modifications might be the cause of the low fiber content of the virions was therefore envisaged. However, after correction of the poly(A) signal, there was no detectable improvement in the fiber copy number of the virions (Table 1 and Fig. 2a). Furthermore, some recombinant viruses with a mutated poly(A) sequence in their fiber gene, such as Ad5/WT-fiber/Xhol, showed normal or nearly normal fiber content (Table 1), suggesting that the poly(A) signal was not implicated directly in the low-fiber-content phenotype. More importantly, when the nucleotide sequence corresponding to the knob domain was reinserted in the fiber gene downstream of the stop codon, as in Ad5/R-ZZwt/r20-Cter, the fiber content of the recombinant virus was not restored to WT levels (Table 1 and Fig. 2a, lane 1). This also excluded the role of untranslated nucleotide sequence overlapping the poly(A) signal at the 3’ end of the fiber gene in the low level of knobless fiber encapsidation.

Intracellular levels of fiber mRNA

The total amount of fiber mRNA was determined in adenovirus-infected cells at 24 and 30 h.p.i. using RPA, a technique designed to measure all of the mRNA species

<table>
<thead>
<tr>
<th>Fiber acronym*</th>
<th>Poly(A) signal sequence†</th>
<th>Knob‡</th>
<th>Fiber copy no. in Ad5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-ligated fibers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-fiber</td>
<td>Normal: AATAAGA-</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td>WT-fiber/Xhol</td>
<td>Mutated: AATaaCTCAGGTAAGA-</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td>R7-knob</td>
<td>Normal: AATAAGA-</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td>RGD-ligated fibers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R7-RGD</td>
<td>Mutated: ACTaaCTCAGGTAAGA-</td>
<td>−</td>
<td>Low</td>
</tr>
<tr>
<td>R7-RGD-knob</td>
<td>Normal: AATAAGA-</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td>ZZwt-ligated fibers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R7-ZZwt</td>
<td>Mutated: AATaaCTCAGGTAAGA-</td>
<td>−</td>
<td>Low</td>
</tr>
<tr>
<td>R7-ZZwt/corr-poly(A)</td>
<td>Corrected: AATaaCTCAGGCGCAAGAATAAAAGA-</td>
<td>−</td>
<td>Low</td>
</tr>
<tr>
<td>R7-ZZwt/r20-Cter</td>
<td>Corrected: AATaaCTCAGG-(from repeat 20 to fiber end)-AATAAAAGA-</td>
<td>−</td>
<td>Low</td>
</tr>
<tr>
<td>R7-ZZwt-knob</td>
<td>Mutated: AATaaCTCAGGTAAGA-</td>
<td>+</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* Schematic representations of WT and recombinant fibers are shown in Fig. 1.
† Sequence at the 3’-terminal end of the fiber gene, after which the WT genome continues. Stop codons are shown in lower case and the WT poly(A) signal in bold italics; the Xhol site is underlined.
‡ Presence (+) or absence (−) of the knob domain in the recombinant fiber protein.
hybridized to a given probe, from unprocessed nuclear precursor mRNA to fully processed mRNA products. The radiolabelled probes used were specific for fiber and hexon mRNAs, the latter serving as an internal control. Differences were observed in the relative levels of fiber mRNA between knob-carrying and knobless fibers, but they did not correlate with the fiber content of virions (Fig. 2b). Moreover, variations within the group of knob-carrying vectors (about twofold between Ad5/WT-fiber/Xho1 and Ad5/R7-knob) were greater than the difference between the groups of knob-carrying and knobless vectors (about 30%). To verify that there was no fragmentation of fiber mRNA at their 3' end, the levels of polyadenylated fiber mRNA species were also measured by real-time PCR on cDNA synthesized from oligo(dT) primers. The values were found to be equivalent for knob-carrying and knobless fiber mRNA (Fig. 2c), indicating that the low-fiber-content phenotype of knobless fiber adenovirus vectors did not result from lower cellular levels of fiber mRNA.

**Fiber protein synthesis and stability**

We then explored the low-fiber-content phenotype of knobless adenovirus virions at the fiber protein level and analysed the rate of fiber synthesis and stability in adenovirus-infected cells using metabolic labelling in pulse–chase experiments. Cells were pulse-labelled with [35S]methionine for 5 min at 21 h p.i. and cellular proteins were analysed after the pulse by SDS-PAGE and autoradiography. As the electrophoretic mobility of WT fiber (62 kDa) and IIIa protein (63 kDa) made quantification of the fiber protein band difficult, comparison was made between short-shafted, knob-carrying (Ad5/R7-RGD-knob; 41 kDa) and knobless (Ad5/R7-ZZwt; 31 kDa) fibers, normalized to the penton base label. The fiber content of Ad5/R7-RGD-knob has been shown previously to be similar to Ad5/R7-ZZwt-knob, with fiber : penton base ratios of 0·6 and 0·7, respectively (Hong et al., 2003). We found a clearcut difference in the rate of synthesis between the two fiber species, with four to five times less incorporation of amino acid in the knobless compared with the knob-carrying fiber (Fig. 3a, b). Fiber protein stability was analysed during a chase period of 9 h after a 45 min pulse at 21 h p.i. (Fig. 3c). A faster rate of fiber degradation was not observed in Ad5/R7-ZZwt-infected cells compared with Ad5/R7-RGD-knob-infected cells, indicating a similar stability for knobless and knob-carrying fibers.

**Fiber protein status**

The whole-cell content of fiber polypeptide was determined in adenovirus-infected cell lysates harvested at different times p.i., using SDS-PAGE and immunoblotting with mAb 4D2.5, which reacts with the common linear epitope
11FNPVYP17 in the fiber tail (Hong & Engler, 1991; Hong & Boulanger, 1995). The pattern of SDS-denatured fiber proteins showed that knobless Ad5/R7-ZZwt fiber was present at significantly lower levels than Ad5/WT-fiber at all time points and even at late times p.i. (72 h p.i.; Fig. 4a). By contrast, cellular levels of its knob-carrying version, Ad5/R7-Zwt-knob, occurred at WT levels at 48 h p.i. (Fig. 4a). The limitation on time-point analysis was due to cytotoxic effects occurring after 72 h.

O-GlcNAc modification has been shown to occur in Ad5 fibers (Mullis et al., 1990) at serine-109 (Cauet et al., 2005), and O-glycosylated fiber reacts with mAb RL2, specific for O-GlcNAc-containing peptides (Mullis et al., 1990). When normalized to their reactivity to mAb 4D2.5, knob-carrying and knobless fibers showed comparable signals to RL2 (data not shown), indicating that both fiber protein species were co-translationally modified by O-GlcNAc transferase with similar efficiency.

The oligomerization status of knobless versus knob-carrying fibers was determined using NDS-PAGE, which separates fiber monomers (a single band at 62 kDa) from trimers migrating as multiple discrete bands at 180 kDa (Mitraki et al., 1999; Novelli & Boulanger, 1991a, b). Fiber trimers were detected in knobless Ad5/R7-ZZwt-infected cells, although with a delay in appearance compared with Ad5/WT-fiber and Ad5/R7-Zwt-knob (Fig. 4b), as in the

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**Fig. 3.** Synthesis and stability of fibers. (a) Autoradiogram of SDS-PAGE analysis of adenovirus-infected cells expressing knobless (lane 2) or knob-carrying (lane 3) fiber after 5 min pulse-labelling with [35S]methionine at 21 h p.i. (b) Histogram corresponding to (a), showing the fiber:penton base protein ratio (mean ± SD, n = 5). (c) Autoradiogram of SDS-PAGE analysis of pulse-chase labelling experiments. Cells were labelled with [35S]methionine for 45 min at 21 h p.i. and radioactivity chased for 0, 3, 6 and 9 h.

**Fig. 4.** Trimerization of fibers. (a) Samples of adenovirus-infected cell lysates expressing WT, knob-carrying or knobless fibers harvested at different times p.i., as indicated above the panel, were heat denatured in the presence of SDS and the fiber content was analysed by SDS-PAGE. (b) NDS-PAGE and immunoblot analysis of the same samples, mixed with SDS-loading buffer without heat denaturation. Fiber was revealed by anti-fiber tail mAb 4D2.5. M, molecular mass markers; m, fiber monomers; t, trimers; *, secondary antibody-reacting cellular protein of 22 kDa used as an internal control. (c) Kinetics of formation of fiber trimers, expressed as the ratio of fiber trimers (as determined in b) to total fiber content (as shown in a).
SDS-PAGE pattern of total fiber content (Fig. 4a). The kinetics of fiber trimer formation showed parallel slopes for knob-carrying and knobless fibers (Fig. 4c), indicating that the trimerization process of our knobless fiber proteins was not altered to detectable levels.

Nuclear localization was investigated by immunofluorescence microscopy using mAb 2A6.36, which recognizes a trimer-dependent epitope in the N-terminal domain of Ad2 and Ad5 fibers (Hong & Engler, 1991). An intense nuclear signal comprised of diffuse fluorescence and brighter speckles was observed in cells infected with Ad5/WT-fiber (Fig. 5a, d) or with the knob-carrying vector Ad5/R7-RGD-knob (Fig. 5b, e). The same type of pattern, although with a lesser intensity, was shown by Ad5/R7-RGD (Fig. 5c, f), indicating that knobless fiber protein self-assembles in trimers, which are transported to the nucleus. This confirmed the SDS-PAGE pattern and excluded a block in trimerization and nuclear import as the major cause of the low fiber content of knobless virions.

**Influence of insertion of additional fiber sequences**

As all recombinant adenoviruses carrying fibers terminated by the knob domain had a normal or nearly normal fiber content (Table 1), we explored the possibility that reinsertion of limited regions of the knob or shaft domain known to be involved in fiber stability might restore the normal fiber content of the virions. We inserted the first 77 aa of the knob between the SpA linker and the Zwt ligands to generate Ad5/R7-401–477-ZZwt, or the TLWT motif and the last 10 aa of the knob downstream of the Zwt ligands, generating Ad5/R7-ZZwt-TLWT and Ad5/R7-ZZwt-572–581, respectively (Fig. 1). The region 401–477 overlaps the β-strand C, in which an Ala→Val substitution has been found to confer a temperature-sensitive phenotype on the fiber (Boudin et al., 1983; Caillat-Boudin et al., 1991). Likewise, a function in fiber assembly has been assigned to the conserved TLWT motif present at the shaft–knob junction and to the C-terminal sequence of the fiber knob (Hong & Engler, 1996). None of these modifications resulted in significant enhancement of fiber quantity in adenovirus-infected cells and corresponding virus progeny (Fig. 6b, lanes 3–5). We also increased the length of the shaft to 10 and 13 repeats and determined whether this positively affected the virus fiber content. Again, no improvement in fiber encapsidation was observed for knobless Ad5/R10-ZZwt and Ad5/R13-ZZwt fibers compared with Ad5/R7-ZZwt (Fig. 6b, compare lanes 6 and 7 with lane 2). By contrast, virions of Ad5/R10-knob and Ad5/R13-knob showed normal fiber content (Fig. 6b, lanes 9 and 10). Thus, the detrimental effect on fiber encapsidation due to the absence of the knob domain could not be rescued by limited sequences from the knob, at least from regions 401–477 and 572–581, or by shaft extension to 13 repeats. Further extension to 22 repeats failed to give viable virions (Magnusson et al., 2002).

**Ectopic expression of WT fiber and fiber encapsidation**

It has been reported that when a recombinant fiber gene was cloned into the E1 region of the adenoviral genome, although the WT fiber gene was maintained in its natural L5 position, the resulting virions had a lower copy number of recombinant fibers than WT fibers (van Beusechem et al., 2000). To determine whether co-expression of WT fiber could restore the knobless fiber protein synthesis and encapsidation to WT levels, we used a similar strategy, but with a difference in the construction of the diploid fiber Ad5 genome and the respective locations of the fiber alleles. WT fiber gene was inserted into the E1 region under the control of the CMV immediate-early promoter, while the gene encoding the knobless fiber was kept in the L5 region. The resulting virus was designated Ad5/R7-ZZwt/E1 : WT-fiber. Knobless Ad5/R7-ZZwt fiber was barely detectable on blots at 24 h p.i. and only became visible at 48 h p.i. (Fig. 6c, lanes 1 and 3). At 48 h p.i., knobless fiber found in Ad5/R7-ZZwt-infected cells was present at levels 10 times lower than WT

![Fig. 5. Immunofluorescence analysis of fiber trimerization and nuclear import.](http://vir.sgmjournals.org)
fiber in Ad5/WT-fiber-infected cells, as normalized to penton base equivalents (Table 2). This confirmed the results of the metabolic labelling of knobless fiber (Fig. 3). At 48 h p.i. in Ad5/R7-ZZ_{wt}/E1 : WT-fiber-infected cells, knobless R7-ZZ_{wt} fiber was not found in larger amounts in double fiber-expressing cells than in single knobless fiber-expressing cells and its level was tenfold lower than that of WT fiber expressed from the same genome (Table 2 and Fig. 6c, compare lanes 3 and 4). When normalized to penton base loads, the viral progeny showed the same low efficiency of encapsidation of knobless fiber in double fiber-encoding Ad5/R7-ZZ_{wt}/E1 : WT-fiber virions as in the single knobless fiber-encoding virions, Ad5/R7-ZZ_{wt} (17 and 20 %, respectively; Table 2 and Fig. 6c, lanes 5 and 6). These results indicated that the lower rate of synthesis of knobless fiber protein was not rescued by its WT allele product.

**DISCUSSION**

The low capsid incorporation of knobless fibers carrying retargeting ligands mentioned in several reports (Henning et al., 2002; Leissner et al., 2001; Magnusson et al., 2001, 2002; van Beusechem et al., 2000) has been attributed to different mechanisms, including differences in the level of fiber protein expression, or/and the lack of flexibility of the fiber construct, depending on the linker separating the fiber shaft and the extrinsic trimerization domain (van Beusechem et al., 2000). Alternatively, the preferential encapsidation of Ad41 short fiber in chimeric Ad5 virions expressing long and short serotype 41 fibers has been suggested to result from its higher affinity for the Ad5 penton base (Schoggins et al., 2003). In this study, our attempts to restore the normal fiber content of our knobless adenovirus vectors through corrections of the fiber untranslated 3\'-sequence or reinsertions of shaft or knob sequences were all unsuccessful. Our results suggested that the cause of the low-fiber-content phenotype of recombinant adenovirus with knobless fiber does not reside at the RNA level and in cis-acting nucleotide sequence(s) in the fiber mRNA, but rather at the protein level and, more specifically, in the fiber C-terminal knob domain.

Our knobless fiber proteins underwent post-translational O-glycosylation, as observed for WT fiber, and assembled into stable trimers that were competent for nuclear import. The only physiological difference between knob-carrying and knobless fibers was the rate of translation of fiber mRNAs, which was slower for the latter. The low efficiency of encapsidation of knobless fibers therefore resulted from the
low abundance of this fiber species within the cell and confirmed a previous hypothesis (van Beusechem et al., 2000). However, the possibility that the low encapsidation efficiency might also be due to subtle changes in the tertiary structure of knobless fiber proteins could not be excluded, although this did not seem to be the major factor, as suggested by the kinetics of appearance of knobless fiber trimers (Fig. 4c).

The data presented in this study indicated that the knob domain exerts a positive control effect on the fiber protein translation machinery, a function that was destroyed by knob deletion and was not restored by reinsertion of partial sequences from the knob. This represents an additional role to the multiple biological properties already reported for the fiber knob, including attachment to cell receptors, intracellular trafficking, endosomal release and virus maturation (Gaden et al., 2004; Legrand et al., 1999; Leissner et al., 2001; Miyazawa et al., 1999, 2001; von Seggern et al., 1999).

The results obtained with our chimeric fiber virus Ad5/R7-ZZwt/E1: WT-fiber suggested that the effect of the knob occurred in cis and could not be compensated for by WT fiber protein provided in trans. In the presence of WT fiber ectopically expressed from a separate promoter in E1, knobless fiber species were still synthesized in much smaller amounts than WT fibers and were poorly encapsidated. The absence of rescue of the negative effect of the knob deletion on fiber protein synthesis and fiber encapsidation by WT fiber co-expressed from the same genome implied that, at least for adenovirus fibers, the translation of each mRNA species was performed by independent machinery within adenovirus-infected cells.

The results presented here emphasize the importance of exploring the consequence on the virus phenotype of attempts to retarget adenovirus vectors to new cellular receptors through genetic manipulations of the fiber, a capsomer that presents severe limitations in terms of ligand functionality and vector viability (Henning et al., 2002; Magnusson et al., 2002). Other sites in the adenovirus capsid that are more tolerant than the fiber for insertion of retargeting ligands, such as pIX, the penton base or the hexon loops, deserve further consideration and therefore extensive studies in the future.

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