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Human adenovirus type 41 possesses different amount of short and long fibers in the virion

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ABSTRACT

To determine the ratio of short fiber (sfiber) to long fiber (lfiber) in human adenovirus type 41 (HAdV-41) virions, sfiber and lfiber were expressed in *E. coli*, quantified, and used as loading standards in Western blot. Densitometric analyses of the standard and target bands indicated that the ratio of sfiber to lfiber in virions was 5.7 ± 0.7. Sfiber-deleted HAdV-41, HAdV-41-DSF-GFP, was constructed, and Western blot analysis showed that the amount of lfiber in HAdV-41-DSF-GFP was about 7.3 ± 1.9 times of that in HAdV-41 virions, confirming a ratio of approximate 6 for sfiber to lfiber in HAdV-41. In HAdV-41-infected cells, mRNAs of the sfiber and lfiber were comparable in quantity, while the expression at protein level was significantly different. Our results suggested an unequal number of short and long fibers, which might result from their differential protein expression during HAdV-41 packaging. The method used here could be extended to quantify other trace proteins.

Introduction

There are more than fifty types of human adenovirus (HAdV) in the Mastadenovirus genus, and they are classified into species HAdV-A to -F (http://www.ictvdb.org/Ictv/index.htm). HAdV-2 and -5 (HAdV-C) are commonly used to study the properties of adenovirus. HAdV-5 has been reconstructed as gene transfer vectors, which are widely used for gene therapy and vaccine development. In contrast to HAdV-C, HAdV-F remains an enigma in some aspects (Tiemessen and Kidd, 1995), although it has been attracting unceasing interest since its discovery.

HAdV-F consists of two members: HAdV-40 and HAdV-41. Several adenoviruses, such as HAdV-2, -5, -12, -18 and -31, can be found in feces samples (Albinsson and Kidd, 1999; Kidd et al., 1982); however, only HAdV-40 and HAdV-41 have been proved to be able to replicate in gastrointestinal tract and cause diarrhea. For this reason HAdV-F was called enteric adenovirus. Gut tropism makes HAdV-F a promising candidate vector for intestinal gene therapy and oral vaccine delivery. When compared to other human adenoviruses, HAdV-F contains a compact genome. The genome of HAdV-41 is approximately 34.2 kb in length, which is 1.7 kb shorter than that of HAdV-5. Interestingly, such a small genome encodes an extra fiber gene: HAdV-F has two fibers. These two fiber proteins form homotrimers, respectively, and protrude externally from different vertices of the virion (Kidd et al., 1993; Yeh et al., 1994). The long fiber (lfiber), just like fibers in other human adenoviruses except for species B, can interact with Coxsackie and Adenovirus Receptor (CAR) (Roelvink et al., 1998), while the receptor of the short fiber (sfiber) is still unknown. It seems that HAdV-41 has equal number of sfiber and lfiber in its virion under electron microscope (Favier et al., 2002). Another unique characteristic of HAdV-F is that its penton base lacks RGD motif (Albinsson and Kidd, 1999). The fiber and RGD motif are the primary and the secondary ligands that adenoviruses use to enter a host cell, and thus, it is understandable why the tropism of HAdV-F is so different from other adenoviruses. In addition, HAdV-41 is acid resistant (Favier et al., 2004; Lu et al., 2009), and it can bind phospholipids and several sphingolipids, which are rich in gastrointestinal mucosa (Favier et al., 2004). However, molecular mechanisms underlying these phenomena are largely elusive. HAdV-F replicates in the gut, but the type of host cells, intestinal epithelial or lymphoid cells, are still undefined.

Study of HAdV-F has been substantially hindered by its property of fastidiousness. Much effort has been devoted to increase the proliferation rate of HAdV-F in vitro. Recently, HAdV-41 has been shown to replicate in 293-ORF6 cells to a level as high as HAdV-5 in 293 cells (Lemiale et al., 2007). We have established a HAdV-41 E1B55K-expressing cell line

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(293TE7), which could be used to amplify both wild type and recombinant HAdV-41 (Zou et al., 2011). A sufficient quantity of viruses makes it possible to study the biological properties of HAdV-41 in a more detailed manner. Here, we attempted to determine the ratio of lfiber to sfiber in HAdV-41 virions using various molecular biological methods.

Results

Amplification and purification of viruses

HAdV-41 was released from 293TE7 cells cultured in twelve 150-mm dishes, and purified by two rounds of ultracentrifugation (Fig. 1a). Finally, 1.8 ml virus suspension was obtained with a particle titer of 5.4×10^{12} vp/ml (viral particle per mililiter). The virus yield reached 2.7×10^4 vp/cell, comparable to that of HAdV-5 replilcated in 293 cells. Icosahedral morphology could be seen clearly under electron microscope (Fig. 1b). Sfiber-deleted recombinant HAdV-41 (HAdV-41-DSF-GFP) could be rescued and similarly amplified in 293TE7 cells. The purified virus stock reached a particle titer of 3.5×10^{11} vp/ml.

Determining the ratio of fibers in HAdV-41 virion by Western blot

There are only 12 trimers of fibers embedded in the vertices of HAdV-41 virion. The content of fibers in total structure proteins of HAdV-41 is too low to be determined directly by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); therefore, the Western blot method was employed.

Intact sfiber (sfiber-P) and lfiber (lfiber-P) were expressed as inclusion bodies in prokaryotic cells (no mutations or tag sequences were introduced). The dissolved inclusion bodies were first quantified with bicinchoninic acid (BCA) method, and then resolved in SDS-PAGE with varied quantities of bovine serum albumin (BSA) as loading standards (Fig. 2a). The loading amounts of BSA and the intensities of corresponding bands were used to establish a standard curve, according to which the concentrations were determined to be 52.8 ng/μl for sfiber-P and 55.6 ng/μl for lfiber-P SDS-PAGE samples, respectively (Fig. 2b). Molarity of sfiber-P SDS-PAGE sample was 1.28 pmol/μl since sfiber-P has a molecular weight of 41,373 Da. Similarly, the molarity of lfiber-P SDS-PAGE sample was determined to be 0.927 pmol/μl (the predicted molecular weight of lfiber-P was 60,627 Da).

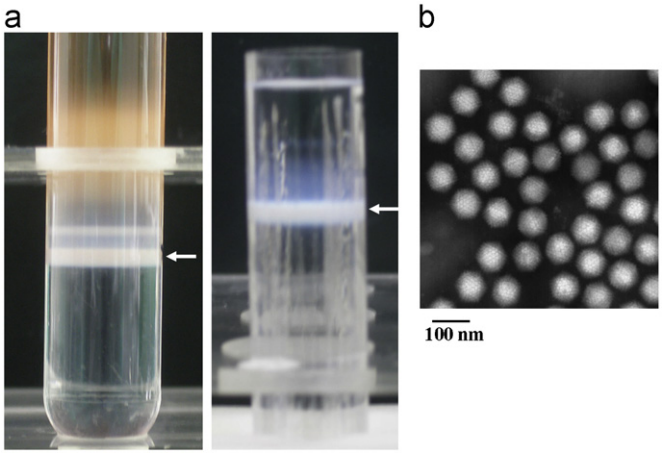

Fig. 1. Amplification and purification of HAdV-41. (a) Wild-type HAdV-41 was amplified in 293TE7 cells, and purified with the cesium chloride method. The virus bands could be clearly seen after the first (left) and second (right) rounds of ultracentrifugation. (b) Typical morphology of adenovirus observed under electron microscope.

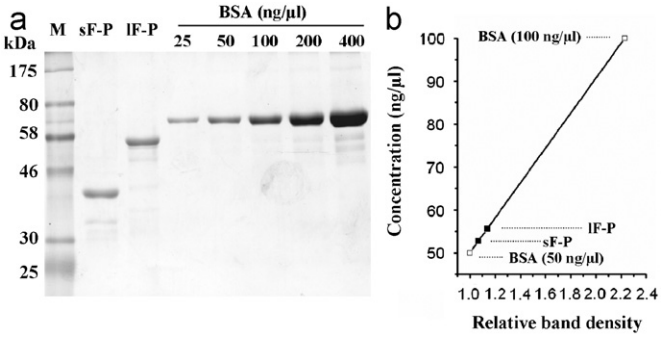

Fig. 2. Quantification of sfiber and lfiber expressed in *E. coli*. Fibers (sfiber-P and lfiber-P) were expressed as inclusion bodies in *E. coli* BL21(DE3) strain. They were dissolved in 2% SDS-1 mM DTT solution, diluted and preliminarily quantified with BCA method. The protein concentrations were adjusted to achieve approximately equal value. (a) The dissolved inclusion bodies were resolved by SDS-PAGE and visualized by Coomassie blue R250 staining. Serially diluted BSA solutions were used as loading standards (10 μl of sample was loaded in each well). (b) Relative band intensity was determined with the software of Gel-Pro analyzer after image digitization. The intensity values of 50 and 100 ng/μl BSA (hollow squares) were used to establish a linear standard curve. The concentration of sfiber-P or lfiber-P (solid squares) could be read from the standard curve according to their relative band intensities. M, Protein Marker (Cat. no. P7708S; NEB, Ipswich, MA, USA); sF-P, sfiber-P; lF-P, lfiber-P.

The SDS-PAGE samples of fibers were further diluted 150, 300 and 600 folds, and used as the loading standards in Western blot to determine the amount of sfiber and lfiber in HAdV-41 virions (Fig. 3a). The concentration of sfiber in the 20-fold diluted HAdV-41 was determined to be 2.44 fmol/μl when the 600- and 300-fold diluted sfiber-Ps were used as standards (Fig. 3b), while that of lfiber in 5-fold diluted HAdV-41 was determined to be 1.81 fmol/μl (Fig. 3c). Therefore, the ratio of sfiber to lfiber in virions should be 5.4 [$=2.44 \times 20/(1.81 \times 5)$]. The same experiments were repeated three times (from protein assay to Western blot), and the results of the other two were 5.2 and 6.5, indicating an average ratio of 5.7 with a standard deviation of 0.7.

Determining the ratio of fibers in HAdV-41 virion using sfiber-deleted recombinant viruses

Point mutations were introduced into the start codon and an internal "GGA" codon of sfiber gene, so the translation of sfiber mRNA was disrupted and only lfiber was expressed in HAdV-41-DSF-GFP virions. Serially diluted HAdV-41-DSF-GFP samples as well as HAdV-41 were loaded for Western blot analysis to visualize fibers and hexon bands. Results from one of the duplicate experiments were shown in Fig. 4. If we accept that both adenoviruses contain equal number of hexon capsomeres and fiber trimers, the number of loaded HAdV-41-DSF-GFP and HAdV-41 could be normalized by comparing the intensities of hexon bands, and then, the difference in amount of lfiber on equal number of virions could be calculated by analyzing the intensities of lfiber bands. After analysis of the result of Western blot, it was found that the number of lfiber on HAdV-41-DSF-GFP was 7.3 ± 1.9 times of that in HAdV-41. If we admit that both viruses have equal fiber trimers on one virion, the ratio of sfiber to lfiber in HAdV-41 should be 6.3 ± 1.9.

Quantifying mature mRNAs of sfiber and lfiber in HAdV-41-infected cells

The SYBR Green real-time PCR was utilized to detect the expression of fiber genes at mRNA level. Human adenovirus genomes encode one late transcription unit (major late) that is processed into several families of late mRNAs. Late mRNAs have a

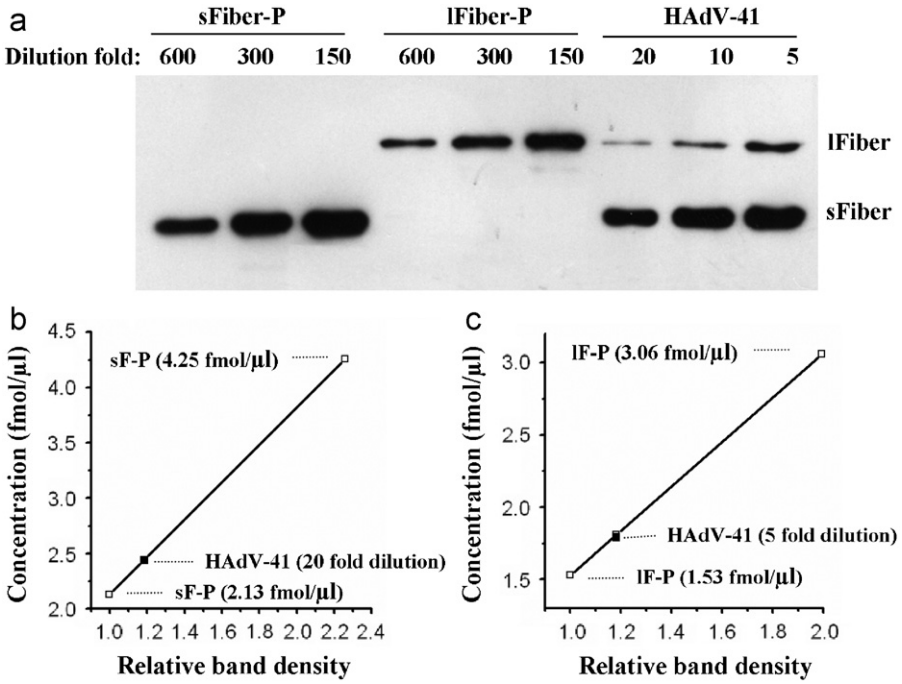

Fig. 3. Quantification of the ratio of sfiber to lfiber in HAdV-41 virion with Western blot. (a) Samples of sfiber-P, lfiber-P and purified HAdV-41 were 2-fold serially diluted and resolved by SDS-PAGE (10 μl of sample was loaded in each well). Fiber bands were visualized by Western blot with an anti-Adenovirus fiber antibody [4D2] (Cat. no. ab3233, Abcam, Hongkong, China). The image was scanned as digital document and analyzed with the software of Gel-pro analyzer 4.0 (Media Cybernetics, Silver Spring, MD, USA). (b) Intensities of sfiber bands were relatively quantified with Gel-pro analyzer where the value of 600-fold diluted sfiber-P band was set as 1. The intensities of 600- and 300-fold diluted sfiber-P bands and the corresponding molarities (hollow squares) were used to establish a linear standard curve. The molarity of sfiber in 20-fold diluted HAdV-41 sample (solid square) could be read from the curve according to its relative intensity. (c) Intensities of lfiber bands were relatively quantified with Gel-pro analyzer where the value of 600-fold diluted lfiber-P band was set as 1. The intensities of 600- and 300-fold diluted lfiber-P bands and the corresponding molarities (hollow squares) were used to establish a linear standard curve. The molarity of lfiber in 5-fold diluted HAdV-41 sample (solid square) could be read from it according to its relative intensity.

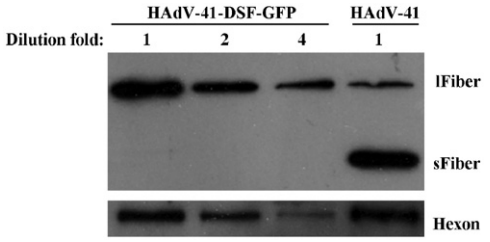

Fig. 4. Quantification of the ratio of sfiber to lfiber in HAdV-41 virion using HAdV-41-DSF-GFP as standards. Purified HAdV-41-DSF-GFP, a sfiber-deleted recombinant HAdV-41, was serially diluted and loaded for SDS-PAGE together with HAdV-41 sample. Fiber bands were visualized by Western blot with an anti-Adenovirus fiber antibody [4D2] (Cat. no. ab3233, Abcam, Hongkong, China), while hexon was stained to show the amount of viruses loaded (anti-hexon antibody, Cat. no. 1401, Virostat, Maine, USA). The image was scanned as digital document and analyzed with the software of Gel-pro analyzer 4.0. The relative amount of lfiber in HAdV-41 lane to that in 4-fold diluted HAdV-41-DSF-GFP was calculated as 0.73, while the relative amount of hexon in HAdV-41 lane to that in 1-fold diluted HAdV-41-DSF-GFP was calculated as 1.09. Therefore, for equal number of virions, the number of lfiber in HAdV-41-DSF-GFP is 5.97 ($4 \times 1.09/0.73$) times of that in HAdV-41.

common characteristic: all of them contain a tripartite leader sequence (TPL) at the 5′ non-coding region. To detect the mature mRNAs, a sense primer was designed using the sequence in the TPL region, while antisense primers were chosen from the coding sequence of sfiber and lfiber, respectively. The genome or unprocessed mRNA could hardly be amplified with these primers possibly due to too long PCR products.

The specificity of PCR was first evaluated with traditional semi-quantitative PCR (Fig. 5a) using cDNA template, which originated from HAdV-41-infected 293TE7 cells. Both bands of sfiber and lfiber could be observed from cycle 23, while the non-specific PCR products started to appear after 6 more cycles (cycle 29). The result suggested an approximately equal abundance of mature sfiber and lfiber mRNA in HAdV-41-infected 293TE7 cells, as well as a negligible non-specific amplification.

The expression of sfiber and lfiber mRNAs in 293 and 293TE7 cells was further quantified with real-time PCR. As shown in Fig. 5b, the mRNA transcription reached its highest level at 24 h post infection (hpi), persisted until 48 hpi, and decreased to a negligible level at 72 hpi. The amount of mRNA produced at 12 hpi was approximately 10 times lower than that at 24 hpi, so the mRNAs transcribed between 24 and 48 hpi substantially affected the expression of fiber protein. At these two time points, the mRNA amount of sfiber was slightly more than that of lfiber in both 293 and 293TE7 cells. The largest difference (1.6 times) was seen in 293 cells at 48 hpi. These results suggested that difference in mRNA transcription contributed little to the different numbers of short and long fibers on HAdV-41.

Evaluating the expression of sfiber and lfiber at protein level in HAdV-41-infected cells

The expression of fiber proteins in cells was investigated further with Western blot. As shown in Fig. 6a, the intensities of sfiber and lfiber bands were notably different in every HAdV-41-infected cell line. It is hard to calculate a precise ratio of fibers from this image since sfiber bands were overexposed to visualize faint lfiber bands. However, when compared with purified virus, it could be concluded that the ratio of fibers expressed in cells was similar to that in HAdV-41 virions (Fig. 6a). If a recombinant virus (HAdV-41-GFP) instead of wild type HAdV-41 was used to infect the cells, the result was also similar (Fig. 6b). The expression of

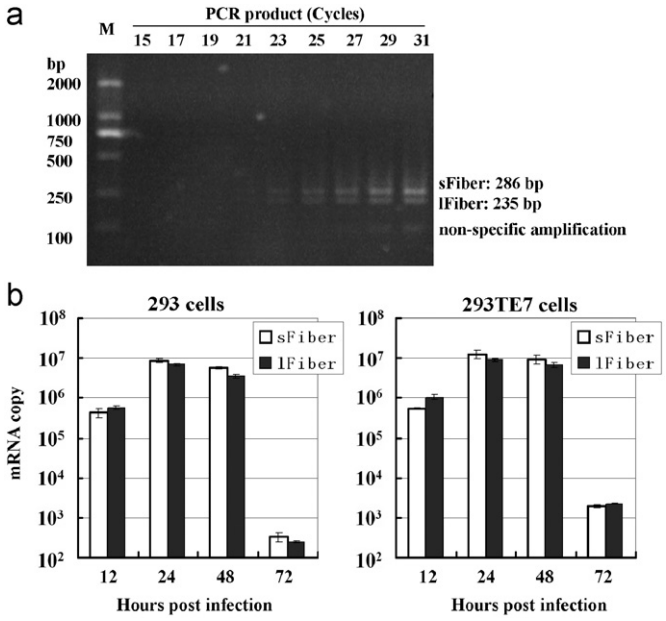

Fig. 5. Comparison of the expression of sfiber and lfiber at mRNA level. Cells were infected with HAdV-41 at an MOI of 100. RNA was extracted, reversely transcribed to cDNA with oligo(dT)18 as the primer, diluted and used as PCR template. (a) Semi-quantitative PCR was employed to compare mRNA expression of fiber genes and to evaluate the non-specific amplification. cDNA originated from 293TE7 cells at 48 h post infection (hpi). Both of sfiber and lfiber bands started to be visible from cycle 23, while non-specific product occurred from cycle 29. The result suggested mRNA amount of the two fibers were comparable and the non-specific amplification could be neglected. (b) SYBR green real-time PCR was further conducted to quantify mRNA copy number of sfiber and lfiber at various time points post infection. The copy number of lfiber was slightly less than that of sfiber. The largest difference could be seen in 293 cells at 48 hpi, where the copy number of sfiber is 1.6 times of that of lfiber.

fibers was then investigated kinetically. As shown in Fig. 6c, no fibers were detected at 12 or 24 hpi, and the ratio of sfiber to lfiber was similar at 48 and 72 hpi, which was identical to that in HAdV-41 virions, although the expression level was a little higher at 72 hpi. Combining the results with these of quantitative RT-PCR, it could be concluded that differential expression of fiber proteins was controlled mainly at the post-transcriptional level.

Discussion

In our studies, it was found that the two fibers in HAdV-41 virions were dramatically different in intensity in Western blot analysis. This phenomenon was unexpected since it was reported that HAdV-41 possessed equal number of short and long fibers on the virion vertices (Favier et al., 2002). The difference might result from different cell lines used to package viruses. 293TE7, a HAdV-41 E1B55K-transfected cell line, was routinely utilized in our laboratory, while 293 cells were employed by Favier et al. (2002). However, the sfiber band with stronger intensity could also be seen in Western blot images from Favier's report, although the authors didn't comment on this phenomenon. The same phenomenon could also be seen in an earlier report (Yeh et al., 1994). The same anti-fiber antibody was used in these two reports and in our experiments, so it was a common phenomenon that short fiber produced a band with much stronger intensity than long fiber did in Western blot analysis.

Another possible explanation is that the anti-adenovirus fiber antibody [4D2] binds sfiber and lfiber with various affinities. The recognized epitope 4D2 was first mapped to the N-terminus of HAdV-2 fiber (Hong and Engler, 1991), and the exact motif was

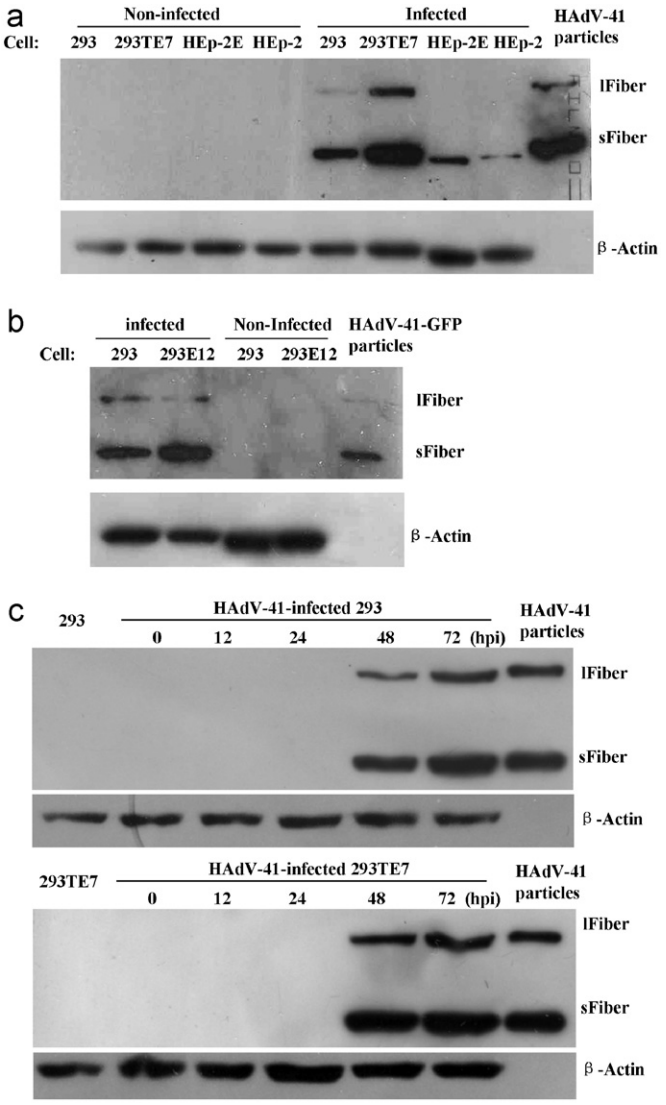

Fig. 6. Comparison of the expression of sfiber and lfiber at protein level. Cells were infected with HAdV-41 (a, c) or HAdV-41-GFP (b) at an MOI of 100. Total protein was extracted at 48 hpi (a, b) or at various time points post infection (c), and subjected to Western blot analysis of fibers expression with anti-Adenovirus fiber antibody [4D2]. Proteins from non-infected cells served as negative controls while purified HAdV-41 or HAdV-41-GFP were used as a positive control. Bands of β-Actin were visualized to show the protein loading. HEp-2E, HEp-2-E1B#4.

further determined to be in a region between residues 10 and 17 (Engler and Hong, 2007; Hong and Boulanger, 1995). The 17 aa at the N-terminus of HAdV-41 fibers was aligned with each other or with that of other human adenovirus species (Fig. 7). The highest degree of similarity between sfiber and lfiber in this region suggested similar affinities of antibody binding between sfiber and lfiber, further supporting the notion that different ratio of sfiber and lfiber exists in HAdV-41 virion.

To address this question quantitatively, intact sfiber and lfiber were expressed in *E. coli* and used as standards to determine the amount of fibers in purified HAdV-41. The prokaryotic inclusion bodies were dissolved and assayed firstly with BCA method. Considering that BCA method could not distinguish target protein from trace impurities, sfiber-P or lfiber-P was further quantified with SDS-PAGE followed by Coomassie blue-staining using BSA as standards. Although the absolute values were different, relative concentration of sfiber-P to lfiber-P matched perfectly between the two methods of protein assays (data not shown). Because the principles of BCA assay and Coomassie blue staining are different,

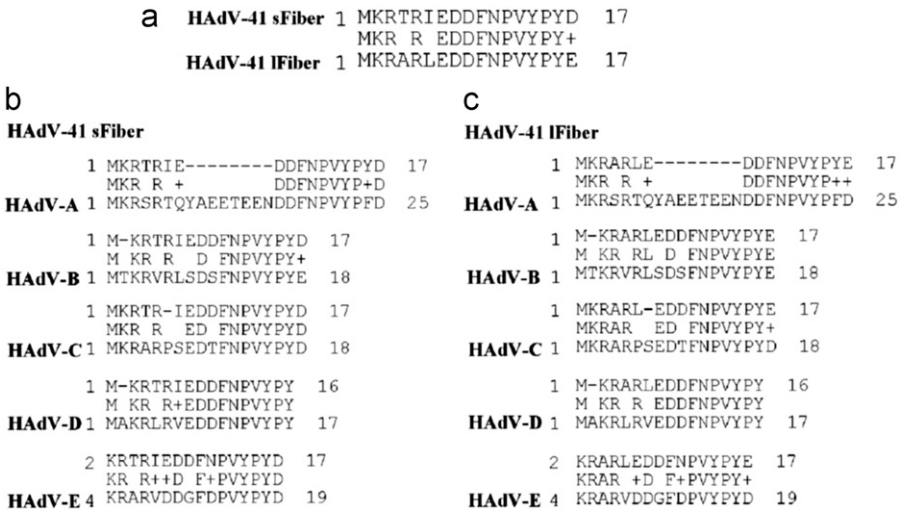

Fig. 7. Sequence alignment of N-terminus of fiber proteins from representative adenovirus types. (a) Sequence alignment of N-terminus of sfiber and lfiber of HAdV-41. (b) Sequence alignment of N-terminus of sfiber of HAdV-41 and fibers of other human adenovirus species. (c) Sequence alignment of N-terminus of lfiber of HAdV-41 and fibers of other human adenovirus species. HAdV-41 sfiber, accession number ADN06466.1; HAdV-41 lfiber, ADN06467.1; HAdV-A (HAdV-12), P36711; HAdV-B (HAdV-35), AP_000601.1; HAdV-C (HAdV-5), AP_000226.1; HAdV-D (HAdV-10), BAG69148; HAdV-E (HAdV-4), AAT97514.1.

the matched results suggested that the relative concentrations of the two proteins were accurate and reliable. Analysis of Western blot revealed a ratio of 5.7 ± 0.7 (sfiber:lfiber) in the virions. These results demonstrated a different ratio of sfiber and lfiber for HAdV-41, which was further confirmed by the sfiber deletion experiments.

Two possible mechanisms could be responsible for the different amount of short and long fibers displayed on HAdV-41 surface. The first mechanism is that sfiber may bind penton base more firmly and then have more advantages in competition with lfiber to construct penton. The N-terminus of fibers is the penton base-binding site, while the basic interaction motif (FNPVYPY) is conserved in nearly all human adenoviruses (Zubieta et al., 2005). It is difficult to verify the first hypothesis because it is almost unfeasible to quantitatively measure the interaction between fiber and penton base. The second possible reason is that the fibers are expressed in different abundance. The one with higher concentration has more chances to bind penton base. The expression of sfiber and lfiber was determined at the mRNA and protein levels. Real-time PCR revealed that the abundance of the two fibers' mRNA was comparable, while Western blot analyses showed that the expression of proteins was significantly different. Regulation of fiber expression at the translational level has also been reported in HAdV-5-infected cells (Henning et al., 2006). Furthermore, the difference in abundance of sfiber and lfiber proteins in virus-infected cells was similar to that in purified HAdV-41 virions (Fig. 6). Therefore, the differential expression of short and long fibers might be the major contributor to the diverse amount of fibers in HAdV-41 virions.

Our findings have possible application in the construction of targeting adenovirus vectors. Fiber is the key factor to determine the tropism of adenovirus. Most human adenoviruses recognize and bind CAR while fibers of HAdV-B use CD46 as the receptor. Recently, new receptors were found for subgroup of HAdV-B and HAdV-37 (HAdV-D) (Nilsson et al., 2011; Wang et al., 2011). It is possible to change or modify adenovector's tropism by introducing either another kind of fiber or further adjusting the ratio of different fibers (Pereboeva et al., 2004; Schoggins et al., 2003). In addition, the strategy of overexpressing target protein prokaryotically and using it to establish a standard curve in Western blot assay could be developed as a convenient and practicable method to quantify trace amount of target protein in a complicated background of other abundant proteins.

Materials and methods

Construction of a short fiber-deleted HAdV-41 plasmid

Backbone plasmid of HAdV-41, pAdbone41, was cut with *Avr*II, and the short fragment (11,360 bp) was ligated to an *Avr*II-digested ampicillin resistant gene-pBR322 origin containing fragment, which was amplified from pMD18-T plasmid (Takara, Dalian, China) with primers 0812DE01 and 0812DE02, to generate pMD-Favr41. pKan was cut with *Bss*HII and *Bst*Z17I, and ligated with an annealed adaptor of 1001PB01 and 1001PB02, which contained *Pac*I and *Bst*BI sites, to generate pKan-PB. Fibers containing fragment (3025 bp) was excised from pMD-Favr41 by *Pac*I and *Bst*BI, and inserted into pKan-PB to generate pKFiber41. Overlap extension PCR was employed to delete the adenine nucleotide in the methionine codon of sfiber with primers of 1001sF01-1001sF04 using pKFiber41 as the template. The PCR product (994 bp) was digested with *Pac*I and *Sac*I, and inserted to the same sites of pKFiber41 to generate pKFiber41DA. pKFiber41DA was excised with *Pac*I and *Bst*BI, and used to replace the fibers region in pMD-Favr41 to generate pMD-Favr41DSF. pMD-Favr41DSF was digested with *Avr*II. The sfiber-deleted fragment (11,358 bp) was used to replace the corresponding region in pAd41-GFP to generate pAd41-DSF-GFP plasmid (pAd41-GFP with deleted sfiber). pAdbone41, pKan and pAd41-GFP were constructed previously (Lu et al., 2009), the primers were summarized in Table 1, and the mutated codons were confirmed by sequencing.

Cell lines and viruses

293TE7 was established in the laboratory and routinely used to amplify HAdV-41 (Zou et al., 2011). 293A, a subclone of 293 cells, was purchased from Invitrogen corporation (Cat. no. R705-07; Invitrogen, Carlsbad, CA, USA). Cell line HEp-2 (ATCC no. CCL-23) was from American Type Culture Collection (ATCC, Manassas, VA, USA). The HAdV-41 E1B55K-transfected HEp-2 cell line, HEp-2-E1B#4, was established previously (Han et al., 2007). Wild-type HAdV-41 (HAdV-41) was isolated from a feces sample (NIVD103)

Table 1
DNA oligos synthesized for PCR or adaptors.

Name	sequence	Length of product (bp)	restriction enzyme site
0812DE01	ggcccctagg atcagctcac tcaaaggcgg taat		*Avr*II
0812DE02	ggcccctagg gtgcgcggaa cccctatttg ttta	1871	*Avr*II
1001PB01	cgcgttaatt aaggccggtt cgaaggt		*Pac*I, *Bst*BI
1001PB02	accttcgaac cggccttaat taa	27	*Pac*I, *Bst*BI
1001SF01	tgcgcgttaa ttaaagttat ctgattaata		*Pac*I
1001SF02	cttcaattct ggttcttttc actgcaagat gaagatcg		–
1001SF03	cgatcttcat cttgcagtga aaagaaccag aattgaag		–
1001SF04	ctaatacgtg agctcgtttt tagttagaca aag	994	*Sac*I
1009SF01	ccccggcata tgaaaagaac cagaattgaa gac		*Nde*I
1009SF02	ccccggctcg agttattgtt cagttatgta gcaaaatac	1185	*Xho*I
1009LF01	ccccggcata tgaaacgagc cagacttg		*Nde*I
1009LF02	ccccggctcg agttattgtt ctgttacata agaaaat	1710	*Xho*I
1009TPL01	gaaagcatcg agccaatcac		–
1009SF03	ggaggagaaa cagtgggaac	286	–
1009LF03	tttagtcccg tgcccagttt	235	–

(Lu et al., 2009), of which the genomic DNA has been cloned and sequenced (Genbank accession no. HM565136). HAdV-41-GFP, an E1-deleted replicating-defective recombinant virus, was described elsewhere (Lu et al., 2009). pAd41-DSF-GFP was linearized by PmeI, and used to transfect 293TE7 cells to rescue sfiber deleted recombinant HAdV-41-DSF-GFP virus.

All cell lines were cultured with Dulbecco's modified Eagle's medium (DMEM) plus 8% fetal bovine sera (FBS; HyClone, Logan, UT) at 37 °C in a humid incubator containing 5% CO_2. For 293TE7 and HEp-2-E1B#4 cells, G418 (Merck KGaA, Darmstadt, Germany) was added at a final concentration of 0.40 mg/ml except in infection experiments. For viral infection, when exponentially growing cells reached 90% confluence, viruses were diluted in fresh DMEM plus 2% FBS at a half volume of that for regular culture and added to the cells. For example, 2.5 ml viruses-medium mixture was used to infect cells grown in a 25-cm^2 flask. At 24 hpi, additional fresh DMEM plus 2% FBS was supplemented to a volume for routine culture. The viruses would not be removed unless specifically mentioned, and the infectious time was calculated from the moment when viruses were added.

HAdV-41 and HAdV-41-DSF-GFP were amplified in 293TE7 cells, purified with two rounds of cesium chloride ultracentrifugation, and preserved in 0.01 M phosphate-buffered saline (PBS) containing 5% glycerol at −80 °C (Ng and Graham, 2002). The particle titer was calculated by determining the concentration of virus genomic DNA, where 100 ng of genome DNA is equivalent to 2.90×10^9 vp. The multiplicity of infection (MOI) was calculated from particle titer.

Electron microscopy

Purified virus was mixed in equal volumes with PBS containing 0.04% BSA, and then dropped on parafilm. A grid covered with carbon support formvar film was floated on the virus suspension for 1 min, stained with sodium phosphotungstate (pH 6.8), exposed to UV radiation for 10 min, and observed under a FEI Tecnai-12 electron microscope.

Prokaryotic expression of short fiber and long fiber of HAdV-41 (sfiber-P, lfiber-P)

Coding sequence of sfiber was amplified by PCR with plasmid pAd41-GFP (Lu et al., 2009) as the template using primers 1009SF01 and 1009SF02 (Table 1), digested with NdeI/XhoI, and inserted into the corresponding multiple cloning sites (MCS) of pET-30a(+) vector (Novagen, Madison, WI, USA). The generated plasmid was named pET-sfiber41. The lfiber carrying plasmid (pET-lfiber41) was constructed similarly using primers 1009LF01 and 1009LF02 (Table 1). pET-sfiber41 or pET-lfiber41 were used to transform *E. coli* BL21(DE3) strain. The expression of sfiber-P and lfiber-P was induced with 0.5 mM isopropyl-D-thiogalacto-pyranoside (IPTG). Inclusion body was collected from bacteria by sonication, washed with PBS containing 1% Triton X-100 and suspended in PBS according to pET system manual.

SDS-PAGE and Western blot

Inclusion bodies of sfiber-P or lfiber-P were dissolved in the lysis buffer (2% SDS, 1 mM DTT, 20 mM Tris–Cl, pH7.6), boiled for 10 min, and centrifuged at 120,000 *g* for 10 min (Lechtzier et al., 2002). The supernatant was collected, diluted four times with the dilution bufffer (1% SDS, 20 mM Tris–Cl, pH7.6) to decrease the concentration of DTT, and preserved at 4 °C. The preserved supernatant was preliminarily quantified with BCA method (Cat. no. 23227; Pierce, Rockford, IL, USA), mixed with equal volume of 2 × SDS loading buffer (200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 100 mM Tris–Cl, pH6.8), boiled again, diluted with 1 × SDS loading buffer to adjust the concentration of fibers to an approximately equal value, and used as the samples for SDS-PAGE or Western blot.

Proteins from whole cells were extracted with RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) plus protease inhibitors (Cat. no. 04693132001; Roche Applied Science, Penzberg, Germany). Proteins from whole cells or purified virus were mixed in equal volumes with 2 × SDS loading buffer, boiled for 5 min. After centrifugation, the supernatants were used for SDS-PAGE or Western blot.

The bands of SDS-PAGE were visualized by Coomassie blue R250 staining, or transferred to Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and visualized with Western blot technique using antibody against adenovirus fiber [4D2] (Cat. no. ab3233; Abcam, Hongkong, China) or hexon (Cat. no. 1401; Virostat, Maine, USA). For proteins from whole cells, human beta-Actin was stained as protein-loading control with rabbit polyclonal antibody (sc-1616-R, Santa Cruz, Delaware, CA, USA). The results of Coomassie blue staining and Western blot were scanned as digital images and the relative quantity of the bands was analyzed with software Gel-pro analyzer 4.0 (Media Cybernetics, Silver Spring, MD, USA). Lanes and bands were selected manually. "Joining valleys" was selected as the Background correction method, while maximum baseline slope was set as 20%.

Real-time PCR

SYBR Green real-time PCR was employed to detect the expression of fiber genes at mRNA level. RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual, treated with DNase I (Takara, Dalian, China), and reversely transcribed into cDNA with AMV reverse transcriptase (Promega, Madison, USA) using oligo(dT)18 as the primer. cDNA solution was diluted and used as the PCR template. Traditional PCR was performed to amplify sfiber41 fragment

(286 bp) with primers (1009TPL01 and 1009SF03) using cDNA from HAdV-41-infected cells as the template. PCR product was recovered from agarose gel, quantified, confirmed by sequencing, 10-fold serially diluted in EASY Dilution (Takara, Dalian, China), and used as template to establish standard curve for real-time PCR. Lfiber fragment (235 bp) was similarly amplified with primers (1009TPL01 and 1009LF03) and 10-fold diluted as standards. Real-time PCR was performed to quantified mRNA encoding sfiber with primers (1009TPL01 and 1009SF03), or lfiber with primers (1009TPL01 and 1009LF03) using SYBR Premix Dimer-Eraser kit DRR091A (Takara, Dalian, China). 293 or 293TE7 cDNA was used as templates in negative control tubes. Specificity of amplification was further evaluated with semi-quantitative PCR (Han et al., 2007; Kinoshita et al., 1992). Briefly, three primers mixture (1009TPL01, 1009SF03 and 1009LF03) instead of two primers was added to the real-time PCR system; PCR was performed in traditional thermocycler, paused every the other cycle at the extension step from beginning of the fifteenth cycle, and resumed after aspirating 2 μl reaction solution from each tube; the collected PCR solution was resolved on ethidium bromide-containing agarose gel electrophoresis, and bands of PCR products were observed using a UV transilluminator and recorded as digital image. Primers were summarized in Table 1.

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