Derivation of a triple mosaic adenovirus based on modification of the minor capsid protein IX

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Abstract

Adenoviral capsid protein IX (pIX) has been shown to be a potential locale to insert targeting, imaging-related and therapeutic modalities by genetic modification. Recent evidences suggested that capsid protein mosaicism could be a promising strategy for improving the utility of Ad vector. In this study, we explored a method to genetically generate triple pIX mosaic Ad serotype 5 (Ad5) displaying three types of pIX on a single virion. pIXs were modified at their carboxy termini with a Flag sequence, a hexahistidine sequence (His6) or a monomeric red fluorescent protein (mRFP1), respectively. Western blotting analysis and fluorescence microscopy of the purified recombinant virions indicated that all three modified pIXs were incorporated into the viral particles. Immuno-gold electron microscopy (EM) further confirmed that three types of pIX indeed co-existed on an individual virion. These results firstly validated a triple mosaic capsid configuration on pIX, and demonstrated the possibility of further radical design.

Keywords:
Adenovirus
Triple mosaic Ad
pIX modification

Introduction

Adenovirus (Ad) is about 70–100 nm in size, non-envelopedicosahedral virus with a capsid of 252 capsomers: 240 hexons forming the faces and 12 pentons at the vertices, each of which bears a slender fiber (Doerfler, 1996). Ad genome is linear, non-segmented double-stranded DNA with a length of 30–36 kb. Ad vectors have been utilized as a basic tool for gene delivery in a variety of studies, and account for one fourth total number of gene therapy clinical trials worldwide thus far (http://www.wiley.com/legacy/wiley-chi/genmed/clinical). The broad usage of Ad is attributable to its ability to infect a wide range of cell types, high efficiency of gene transfer, ability to incorporate large DNA inserts into its genome, and also efficient methods available for generation of recombinant virions. In regard to the most commonly used Ad serotypes for gene delivery, Ad2 and Ad5 from subgroup C, binding of the globular knob domain of the Ad fiber protein with primary cellular receptor, coxsackievirus group B and adenovirus receptor (CAR), has been identified as the initial step of infection in in vitro studies (Bergelson et al., 1997; Roelvink et al., 1998; Tomko et al., 1997). After administrated in mice, monkeys and pigs as a viral vector, wild type Ad or tropism unaltered recombinant Ad is mainly distributed in the liver, lung and spleen (Breidenbach et al., 2004; Torres et al., 1996; Xin et al., 2005); however, the distribution is highly dependent on animal species and administration methods, e.g. intramuscularly, intravenously or intraperitoneally.

To fulfill gene delivery to CAR-deficient cell types and circumvent host neutralization of Ad vectors, modification on capsid proteins has been explored by genetically incorporating peptide ligands on fiber, penton base and hexon (Dmitriev et al., 1998; Roy et al., 1998; Vigne et al., 1999; Wickham et al., 1996a,b; Wu et al., 2002). However, major capsid proteins are crucial for Ad assembly and stability, and their structural properties only allow for incorporation of constrained heterologous ligands, which apparently have size limitation when added to the C terminus and HI loop of fiber (Belousova et al., 2002; Wickham et al., 1997) and hexon L1 loop (Matthews et al., unpublished data). pIX is expressed at delayed early stage with a molecular weight of 14.3 kDa, and is a minor capsid protein associated with group-of-nine (GONs), acting as a stabilizing cement via its N-terminal domain interaction (Furcinitti et al., 1989; Parks, 2005; Rosa-Calatrava et al., 2001; Vellinga et al., 2005). pIX is considered not required for virus assembly (Colby and Shenk, 1981); however, it is essential for thermotolerance and packaging of full length genomes (Ghosh-Choudhury et al., 1987). Recent Coyo-EM data (Marsh et al., 2006; Saban et al., 2005, 2006), structural and functional studies of pIX (Akali et al., 1999; Rosa-Calatrava et al., 2001) suggested a theoretical possibility that the C terminus of pIX, which either binds on the capsid surface or extends outward from the capsid, can tolerate considerable modification and be utilized as an anchor for the addition of heterologous ligands to Ad particles. This thought was further demonstrated by several studies on Ad tropism alteration (Dmitriev et al., 2002; Vellinga et al., 2007; Zakhartchouk et al., 2004), in vivo imaging and tracking of physical Ad
particles (Le et al., 2004, 2006; Meulenbroek et al., 2004) and cancer therapeutics (Li et al., 2005) via genetically incorporating corresponding heterologous polypeptides on pIX C terminus.

Therefore, pIX is an attractive candidate for the placement of functional motifs, and based on previous studies of pIX modification, we hypothesize that pIX may be utilized as a locale to accommodate multiple functional motifs on a single virion. To test this hypothesis, we attempted to incorporate three types of pIX into a single virion via genetic modification. We created a recombinant type 5 Ad carrying three types of genetically modified pIXs, each of which contained a distinct tag at its C terminus. Using Western Blotting analysis and immunogold labeling electron microscopy, we demonstrated that three types of pIX were incorporated into the virus and could coexist on a single virion. This is, to our knowledge, the first derived triple mosaic Ad, suggesting the possibility of further radical capsid design for simultaneously employing multiple functional modalities.

Results

Generation of genetically pIX-modified Ad5 vectors

Since pIX has been shown to be an incorporation site of heterologous polypeptide ligands on human adenovirus (Ad) capsid without perturbation of viral viability, capsid stability or loss of peptide functionality (Dmitriev et al., 2002; Le et al., 2004, 2006; Matthews et al., 2006; Vellinga et al., 2004), it is possible that multiple pIXs carrying different ligands can be incorporated and presented in a single virion. To investigate this possibility, and test whether three types of pIXs can be efficiently incorporated into Ad, we constructed E1/E3-deleted Ad5 vector carrying three modified pIX genes containing Flag, hexahistidine (His$_6$) or monomeric red fluorescent protein (mRFP1) sequences at their 3’ ends immediately before the stop codons. To minimize internal homologous recombination among modified pIX genes, we employed human cytomegalovirus (CMV) immediate-early promoter to drive IX-Flag gene expression and simian virus 40 (SV40) early promoter to drive IX-His$_6$ gene expression, respectively. These two pIX-expression cassettes were inserted upstream of the IX-mRFP1 gene driven by its native promoter (not shown here). (C) Amino acid residues of Flag and His$_6$ polypeptides and mRFP1 protein (incomplete) that were incorporated into the C terminus of pIX.

![Fig. 1](image-url)

**Fig. 1.** Schema of Ad pIX modification. (A) Structural diagram of Ad5 vector with triple modifications. Flag, His$_6$ polypeptides and mRFP1 protein were incorporated in the C terminus of pIX. (B) Constructs of modified pIX genes in genomes of Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1. IX-Flag and IX-His$_6$ were driven by CMV and SV40 promoter, respectively, and inserted in Ad E1 region. “En” stands for SV40 enhancer. IX-mRFP1 was located at native region of pIX gene, driven by its native promoter (not shown here). (C) Amino acid residues of Flag and His$_6$ polypeptides and mRFP1 protein (incomplete) that were incorporated into the C terminus of pIX.

![Fig. 2](image-url)

**Fig. 2.** Western blotting analysis of Ad vector containing triple pIX modifications. $10^{10}$ VPs of CsCl-purified Ad5-IXFlag-IXHis$_6$-IXmRFP1 were subjected to SDS-PAGE. The separated proteins were probed with rabbit anti-pIX (lane 1), mouse anti-His$_6$ (lane 2) or mouse anti-Flag (lane 3) antibody, and developed with AP-conjugated goat anti-mouse or anti-rabbit secondary antibodies. IX-mRFP1 contains a Flag peptide in the C terminus of pIX when originally constructed (Le et al., 2006). The number on the left indicated the molecular mass in daltons. The (*) symbol was a non-specific band.
Incorporation of modified pIXs in Ad capsid

Western blotting analysis was used to confirm the incorporation of three heterologous pIXs into viral capsid. The CsCl-purified viral particles of Ad5-IXFlag-IXHis6-IXmRFP1 were denatured by boiling and the capsid proteins were dissolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The probing of separated viral proteins of Ad5-IXFlag-IXHis6-IXmRFP1 using mouse anti-Flag, anti-His6 antibodies and a rabbit anti-pIX antibody detected the presence of protein bands with molecular weights of 15 kDa, 21 kDa, and 50 kDa, corresponding to IX-His6, IX-Flag, and IX-mRFP1 fusion proteins, respectively (Fig. 2). The presence of IX-mRFP1 protein was further confirmed by fluorescence microscopy on intact viral particles. Purified virions were directly visualized under a fluorescence microscope to detect the red fluorescent signal of mRFP1 protein, and Ad5-IXmRFP1, Ad5-IXFlag-IXmRFP1, and Ad5-IXFlag-IXHis6-IXmRFP1 viruses were demonstrated to contain mRFP1 protein in viral capsid (Fig. 3). Of note, two mosaic viruses (Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1 in panel C and D, respectively) had less fluorescent signal compared to single pIX-modified virus (Ad5IXmRFP1 in panel B), indicating that the incorporation levels of IX-mRFP1 fusion protein were lower in the two mosaic viruses.

Presentation of modified pIX on virion capsid

To test whether or not the polypeptides incorporated into the C terminus of pIX were presented on viral surface and accessible to their specific antibodies, we performed enzyme-linked immunosorbent assays (ELISAs) in which mouse anti-Flag, anti-His6 antibodies and rabbit anti-RFP antibodies were used to detect three modified pIXs, respectively. The control Ad5 containing wild type pIXs was not recognized by any of these antibodies. The anti-Flag antibody recognized all three pIX-modified Ads since Flag tag was contained in IX-Flag and IX-mRFP1 fusion proteins. Ad5IXmRFP1 showed the highest level of interaction with anti-Flag antibody, and Ad5-IXFlag-IXmRFP1 had slightly weak interaction, while Ad5-IXFlag-IXHis6-IXmRFP1 had the lowest binding (Fig. 4A). The anti-His6 antibody moderately bound to Ad5-IXFlag-IXHis6-IXmRFP1 (Fig. 4B). These results demonstrated that Flag, His6 epitopes introduced into the pIX C termini were displayed on the surfaces of these pIX-modified Ads and accessible for recognition by antibodies. However, anti-RFP antibody did not bind Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1 as revealed by Fig. 4C, while efficiently bound to Ad5IXmRFP1. This could be due to that mRFP1 were masked by other epitopes (e.g Flag or His6) in the heterotrimeric pIX protein, or the low incorporation rate of IX-mRFP1 proteins in Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1, which was under the limit of the ELISA detection.

CAR-dependent binding of pIX-modified adenovirus

Adenovirus has been shown to bind its primary cellular receptor CAR as the initial step of infection via its fiber knob protein. To examine whether the modification of pIX in Ad capsid interferes with the interaction, we performed the binding assay in 293 cells in the presence or absence of soluble CAR (sCAR) protein, which was used as competitive inhibitors. The presence of sCAR (200 μg/ml) blocked >90% cell binding of Ad5, Ad5IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1, >70% of Ad5-IXFlag-IXmRFP1 (Fig. 5A), indicating that the cell binding of all pIX-modified Ad5 is still CAR-dependent.

Infectivity of pIX-modified Ad5 vectors

Since capsid modification may affect the native infectivity of Ad5, we carried out infection assay in four CAR positive cell lines: 293, A549, GH329 and HeLa. Because there is no appropriate reporter gene in the pIX-modified Ads, we could not perform traditional infectivity assay based on transgene expression, e.g. luciferase activity. Nonetheless, we attempted to use the total viral genomes transferred into cells following 2-hour incubation as an assessment of infectivity. After infection, the cells were harvested by trypsin/EDTA and the total DNA

In Fig. 3, Direct visualization of pIX-modified Ad vector by fluorescence microscopy. 5×10^9 VPs of CsCl-purified Ad were mounted on a microscope slide and examined under a fluorescence microscope with a ×100 objective using oil immersion. (A) Control Ad5 with wild type pIX; (B) Ad5IXmRFP1; (C) Ad5-IXFlag-IXmRFP1; (D) Ad5-IXFlag-IXHis6-IXmRFP1. The thick arrows indicate heavily aggregated viral particles and the narrow arrows indicate slightly aggregated or single viral particles.
Thermostability of pIX-modified Ads

pIX is a cement protein and stabilizes Ad capsid. This raised the question that whether the modification on pIX could affect the stability and structure integrity of the viral particle. Thus we compared the thermostabilities of pIX-modified Ads and control Ad5. The remaining titer of Ad5-IXFlag-IXmRFP1, which contains double pIX modifications, was severely reduced when incubated in medium (P = 0.049) and in PBS (P = 0.016). The remaining titer of Ad5-IXFlag-IXHis6-IXmRFP1, which contains triple pIX modifications, was slightly reduced when incubated in medium (P = 0.081) and in PBS (P = 0.13). The results show that the thermostability of pIX-modified Ad5 was compromised, which was consistent with an increased VP/PFU ratio observed in pIX-modified viruses (98, 387, 391, and 245 for control Ad5, single, double, and triple pIX-modified Ad5, respectively).

Growth kinetics of pIX-modified Ad5

During the generation of Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1, we noticed that it took longer for transfected 293 cells to induce complete CPE compared with unmodified Ad5 (data not shown), indicating that the modification on pIX was likely to affect virus packaging. To obtain a quantitative understanding of this effect, growth kinetics of the pIX-modified viruses were studied and compared with that of wild type Ad5. Fig. 6C showed that wild type control Ad5 induced a full CPE and produced a maximum yield of viral progeny 3 days post infection; however, even though all pIX-modified

were extracted for the measurement of total Ad5 genome copy number by quantitative real-time PCR (Q-PCR). All pIX-modified viruses exhibited similar infectivity and showed no significant difference among each other (Fig. 5B). In A549 and GH329 cells, pIX-modified viruses showed comparable infectivity with control Ad5; nevertheless, in 293 and HeLa cells, infectivity of pIX-modified viruses was approximately one half of the control Ad5.

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Immunoelectron microscopy

cells were infected with Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-
modifications (Table 1). The Ad containing single pIX modification (Ad5IXFlag, Ad5IXHis6, and Ad5IXmRFP1) were recognized by corresponding primary and secondary antibodies (Figs. 7, B–D). The Ad containing triple pIX modifications (Ad5-IXFlag-IXHis6-IXmRFP1) was recognized by anti-Flag and anti-RFP antibodies, and labeled with 10 and 25 nm gold particles (Fig. 7E). The Ad containing triple pIX modifications (Ad5-IXFlag-IXHis6-IXmRFP1) was recognized by anti-Flag, anti-His6, and anti-RFP antibodies and labeled with 10, 18 and 25 nm gold particles as shown in Fig. 7F. The results showed that three types of modified pIXs could coexist on one single viral particle. Since a Flag tag is also present at the N terminus of mRFP1 protein, we cannot exclude the possibility that the all Flag epitopes detected in EM experiments came from IX-mRFP1 protein in the double and triple pIX-modified viruses. Nonetheless, this possibility is very rare because incorporation rate of IX-Flag protein was much higher than that of IX-mRFP1 protein as shown in Western blotting of viral particles.

Generation of triple mosaic Ad by co-infection

A co-infection strategy has been employed by Takayama et al. (2003) in generating mosaic Ad, in which two Ad genomes containing distinct serotypes of fiber were co-transferred into 293 cells. In this situation, both fibers were expressed in 293 cells and assembled into virion at random, resulting in fiber mosaic Ad. We therefore utilized this strategy as an alternative method to prove the principle of triple mosaicism on pIX. By co-infecting 293 cells with Ad5IXFlag, Ad5IXHis6 and Ad5IXmRFP1, each of which contains a single type of modified pIX, three IX-ligand fusion proteins were expressed for the production of viral progenies. The results showed that all kinds of double and triple pIX mosaic Ads were successfully generated by co-infecting 293 cells with Ad5IXFlag, Ad5IXHis6 and Ad5IXmRFP1 in corresponding combinations (Fig. 8), indicating that pIX mosaicism is a realizable feature of Ad and co-infection strategy is also an effective and efficient to generate pIX mosaic virus.

Discussion

Adenovirus based viral vector has been utilized in a large number of gene therapy studies via a variety of modifications. Recent evidences suggested that incorporation of functional motifs into Ad capsid has several advantages over conventional gene therapy strategies which express transgenes after infection. For examples, with regard to viral vector imaging, the expression of reporter genes can only provide indirect information of virus localization and replication, which is determined by transcriptional and translational activity of that particular transgene in the target cells, and possibly cause non-specific expression in non-target cells. However, if linked on Ad capsid, polypeptides function directly after viral infection with no need of expression. This property is especially important for transducing certain cell types where promoter activity is low. This thought was explored in the study of monitoring oncolytic adenoviruses in vivo (Le et al., 2006), where the physical trafficking and distribution of Ad were under observation with an imaging modality incorporated on pIX. The C terminus of pIX was suggested to be a potential local to incorporate functional modalities in several studies. Besides the imaging motif (Le et al., 2006), thymidine kinase can also be incorporated in this locale with its native function maintained (Li et al., 2005), suggesting Ad vector’s potentials in oncolysis and cancer therapy. Previous studies (Campos and Barry, 2006; Kurachi et al., 2007) suggested that transductional targeting via incorporating targeting ligand on the C terminus of pIX usually is not as effective as doing thus on fiber knob, partially due to its relatively low accessibility by virtue of the topology and dimensional arrangement of pIX proteins within GONs and steric hindrance effect from fibers. This issue may be circumvented by lifting...
Fig. 7. Immuno-gold electron Microscopy on pIX-modified Ads. Viruses were loaded onto EM grids, probed with gold nanoparticle conjugated antibodies, and observed under electron microscope at 60 kV. (A) Ad5; (B) Ad5IXFlag; (C) Ad5IXHis6; (D) Ad5IXmRFP1; (E) Ad5-IXFlag-IXHis6-IXmRFP1. Primary and gold-conjugated secondary antibodies employed were following: mouse anti-Flag, goat anti-His6, and rabbit anti-RFP primary antibodies were used for detecting 3 epitopes; 10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit secondary antibodies for gold labeling. Solid thin arrows point 10 nm gold particles, empty arrows point 18 nm gold particles and solid thick arrows point 25 nm gold particles. Scale bars in each panel represent 50 nm in length.

Fig. 8. Generation of pIX mosaic Ad5 by co-infection. 293 cells were co-infected with pIX-modified Ads (each virus carries one type of pIX) in the following combination at MOI of 20 VP/cell: A, Ad5IXFlag and Ad5IXHis6; B, Ad5IXFlag and Ad5IXmRFP1; C, Ad5IXHis6 and Ad5IXmRFP1; D and E, Ad5IXFlag, Ad5IXHis6 and Ad5IXmRFP1. CsCl-purified viral particles were stained with corresponding primary and gold-conjugated secondary antibodies as following: mouse anti-Flag, goat anti-His6, and rabbit anti-RFP primary antibodies were used for detecting 3 epitopes; 10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit secondary antibodies were used for gold labeling. Solid thin arrows point 10 nm gold particles, empty arrows point 18 nm gold particles and solid thick arrows point 25 nm gold particles. Scale bars in each panel represent 50 nm in length.
the targeting ligand toward the hexon top via alpha-helical spacers to improve accessibility as illustrated by Vellinga et al. (2004), although this hypothesis is under debate (Campos and Barry, 2006; Kurachi et al., 2007). In addition, better understanding of organ and tissue's physical barriers makes it possible to deliver viral vectors in vivo more efficiently than before by utilizing various transcytosis machineries (Bobardt et al., 2007; Bomsel, 1997; Di Pasquale and Chiorini, 2006; Tang et al., 2007; Zhu et al., 2004), which needs an extra relaying motif for transcytosis besides the targeting ligand against the receptor of destination. On the other hand, development of viral vectors may also be favored in terms of efficacy and reliability by therapeutic synergism and imaging redundancy, both of which need more than one incorporation site to accommodate the synergistically therapeutic motifs or an extra imaging motif on the viral capsid.

In this proof-of-principle study, we explored the possibility of incorporating three different pIX proteins into a single Ad5 virion capsid by replacing the native pIX gene with three expression cassettes encoding three modified pIX proteins containing Flag, Hiŝ6 polypeptides or mRFP1 protein at the C terminus, respectively. The Ad containing double or triple pIX modifications, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1, were successfully rescued. The three modified pIXs were incorporated into viral capsid and accessible to antibodies. By labeling pIX-modified viruses with three tag-specific antibodies which were conjugated with gold nanoparticles in three different sizes, we have showed that two or three heterologous IX proteins could coexist on a single virion of Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis6-IXmRFP1, respectively.

However, pX-modified Ads appeared more thermolabile than control AdS as shown in the heat inactivation assay (Figs. 6A and B), which is not unexpected since pX plays an important role in Ad stability. Of note, Ad5-IXFlag-IXHis6-IXmRFP1 seems to be more thermostable than Ad5XmRFP1 and Ad5-IXFlag-IXmRFP1. This can be explained by that the capsid of triple pIX-modified viruses contains more IX-Flag and IX-His6 proteins, which are small in size and do not affect the cement function of pIX and overall stability of Ad5 virion (Dmitriev et al., 2002). In the growth kinetics experiment (Fig. 6C), the functional multiplicity of control AdS, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1, Ad5-IXFlag-IXHis6-IXmRFP1 were 0.050, 0.013, 0.013, 0.020 PFU/cell, respectively. Thus, the 2-log difference of recovered viral progenies after 24 h between control AdS and pX-modified virus could hardly be explained by different initial infection. This, together with the delayed full CPE formation in pX-modified viruses, suggested that modification on pX had adverse effect on viral replication and/or assembly. These data was consistent with previous studies, in which deletion, mutation and modification of pX was suggested to perturb Ad's stability (Colby and Shenk, 1981; Dmitriev et al., 2002; Ghosh-Choudhury et al., 1987; Rosa-Calatrava et al., 2001; Vellinga et al., 2005). In particular, pX whole protein was essential for genome packaging and stability, and residues 13–15 and 22–28 at the N terminal were vital since deletion of these residues made viruses seriously thermolabile. The central region and C-terminal domains seemed to be trivial in this regard because point mutations (L114P and V117D) and deletions on residues 60–72 (alanine stretch)100–114 (large part of leucine repeat) neither caused problems in pX incorporation nor viral thermolability, arguing for a good tolerance of modification in these two regions. However, the properties of heterologous peptide per se, such as structure, size and electric charge, may affect the stability (Dmitriev et al., 2002). In addition, pX was suggested to be involved in virus-induced nuclear reorganization and inhibiting cellular antiviral responses (Rosa-Calatrava et al., 2001; Rosa-Calatrava et al., 2003), and act as a transcriptional activator (Lut et al., 1997), although the significance is under controversy (Sargent et al., 2004). Therefore, structural compromise on leucine repeat at the C terminus and alanine stretch at the central region of pX may also be a cause of the delayed CPE induction by pX-modified Ads.

Three heterologous pIXs were incorporated into viral capsid as revealed by western blotting analysis on lysed viral protein as well as fluorescence microscopy, ELISAs, and immunogold EM on purified viral particles. Nevertheless, these data also suggested that three types of pIX were not equally incorporated. In Western blotting analysis, IX-Flag protein appeared to be the most and IX-mRFP1 seemed to be the least. Fluorescence microscopy, ELISA and immunogold EM also suggested possible low incorporation rate of IX-mRFP1 protein. The unequivocal stoichiometry of IX proteins could be by virtue of their synthesis since three modified pIX genes were driven by three different promoters (CMV, SV40, and pIX native promoter). These three promoters apparently had different activity since protein expression of modified pIXs in Ad infected cells were quite different both in time and strength (data not shown). In addition, the properties of modified pIX such as size, charge and conformation could also cause aggregation and incompatibility, and interfere with viral assembly (Ugai et al., unpublished data).

By labeling the three types of pIXs with gold nanoparticles in three different sizes, EM provided a direct evidence of the coexistence of heterologous IX–ligand fusion proteins in viral capsid, and further demonstrated that the triple mosaicism could be feature of a single virion rather than mixed viral populations. It is noteworthy that, although there are 240 copies of pIX molecules in each viral particle, only a few gold nanoparticles were bound on Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis6-IXmRFP1 virus. This is probably due to spatial hindrance effect of gold particles, and we chose big gold particles for easier differentiation, which have relatively high spatial hindrance effect against each other during staining. Besides, the low efficiency of triple staining may be the reason of the scattered staining pattern shown in the figure. Therefore, it is not unexpected that the frequency of triply-labeled Ads is low that only about 2% viral particles were positively stained with all three gold particles. Taken together, we have demonstrated for the first time a triple mosaic capsid configuration based on pX modification, and validated the possibility of further radical engineering in pX.

Materials and methods

Antibodies

The pX-specific antibody was a kind gift from Dr. I. Dmitriev (Gene Therapy Center, University of Alabama at Birmingham). The mouse anti-His6 monoclonal antibody was purchased from Qiagen (Valencia, CA.). The goat polyclonal anti-His6 antibody was purchased from Abcam (Cambridge, MA.). The anti-Flag M2 monoclonal antibody was purchased from Sigma (St. Louis, MO.). The rabbit polyclonal anti-RFP antibody was purchased from Chemicon (Temecula, CA.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies, or alkaline phosphatase (AP)-conjugated goat anti-mouse, goat anti-rabbit secondary antibodies, and electron microscopy (EM) grade 18 nm colloidal gold-conjugated donkey anti-goat were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA.). EM grade 10 nm gold-conjugated donkey anti-mouse, EM grade 25 nm gold-conjugated donkey anti-rabbit secondary antibodies were purchased from Electron Microscopy Science (EMS, Ft. Washington, PA.).

Cells

The human embryonic kidney cell line (293) transformed with Ad5-E1 DNA, human lung carcinoma (A549) cells and human cervix epithelial carcinoma cells (HeLa and GH329) were purchased from American Type Culture Collection (ATCC, Manassas, VA.). All cell lines were cultured in Dulbecco's modified Eagle medium-Ham's F12 (50/50) medium (Sigma) containing 10% fetal calf serum (HighClone, Logan, Utah), 2 mM l-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and grown at 37 °C in a 5% CO2 humidified incubator.
Construction of recombinant plasmids

The E1/E3 deleted Ad5 genome containing three modified pIX genes were constructed as following sequentially:

(i) Generation of shuttle vector carrying two modified pIX genes, pShuttle-IXFlag-IXmRFP1. The IX-Flag open reading frame (ORF) was obtained by PCR on the template of an IX-flag containing shuttle plasmid named pShuttleXFlagNebl (Dmitriev et al., 2002), using primers KpnI (s), 5' - GGATCC ATGAGCACCAAC-3' and HindIII (as), 5’ - CCC AAGCTT CTACCGCGCGGAAAACCCTATAA - 3’, with corresponding recognition sequence underlined. Then the PCR product was digested and cloned into pShuttle-CMV plasmid (Stratagene), resulting in pShuttle-IX-CMV-Flag-pA. The expression cassette CMV-IX-Flag-pA was amplified by PCR with primers NotI (5’), 5’ - GCAAGGT GCCGCCGCGAATATCTAGACACGATC - 3’, and HindIII (as), 5’ - GGCGC GTGCAC TAACTAAGCCTTGGATGAGTTTGGAACG - 3’, resulting in a shuttle vector containing two modified pIX genes: pShuttle-IXFlag-IXmRFP1.

(ii) Generation of the final shuttle vector containing three modified pIX genes, pShuttle-IXFlag-IXHis6-IXmRFP1. To incorporate sequence encoding six consecutive histidines into the 3’ end of the pIX gene (pIX-IXmRFP1), two primers HindIII (5’), 5’ - CCC AAGCTT ATGAGCACCAACACTTGGATGAGTTTGGAACG - 3’, and XbaI (as), 5’ - GCC TCCTAGA TTAATGATCTGATAGTGAGTTTGGAACG - 3’, and subcloned into pShuttle-IX-ITRFP1 (Li et al., 2006), resulting in a shuttle vector containing two modified pIX genes: pShuttle-IXFlag-IXmRFP1.

(iii) Generation of pIX-modified adenoviral genomes by homologous recombination in Escherichia coli (He et al., 1998). The shuttle vector pShuttle-IXFlag-IXmRFP1 or pShuttle-IXFlag-IXHis6-IXmRFP1 was linearized with Pmel restriction enzyme and homologously recombined with pAdEasy-1 (Stratagene, La Jolla, CA) in electrocompetent BJ5183-Ad1 (Stratagene). The generated adenoviral genomes contain two (IXFlag-IXmRFP1) or three (IXFlag-IXHis6-IXmRFP1) modified pIX genes in E1 region. The constructs of resultant Ad plasmids pAd5-IXFlag-IXmRFP1 and pAd5-IXFlag-IXHis6-IXmRFP1 were confirmed by restriction digests and sequencing.

Virus rescue, propagation and purification

pAd5-IXFlag-IXmRFP1 and pAd5-IXFlag-IXHis6-IXmRFP1 were linearized with PacI restriction enzyme; then the large fragments (32.3 kb and 33.4 kb, respectively) were purified and transfected into 293 cells grown in 25 cm² flask with Superfect (Qiagen). The cells were collected when evident cytopathic effect (CPE) were observed, and disrupted by four freeze and thaw cycles. The lysates were centrifuged at 3000 ×g for 5 min at 4 °C to move the cell debris. The released viruses in the supernatant were subsequently used for further propagation until sufficient 293 cells were grown in 25 cm² flask with Superfect (Qiagen). The cell debris were collected when evident cytopathic effect (CPE) were observed, and disrupted by four freeze and thaw cycles. The lysates were centrifuged at 3000 ×g for 5 min at 4 °C to move the cell debris. The cell debris containing the viruses were loaded on the top of a 1.33/1.45 CsCl step gradient and centrifuged at 55,000 ×g for 3 h at 4 °C. Lower band containing infectious particles were re-centrifuged on another 1.33/1.45 CsCl step gradient at 100,000 ×g for overnight at 4 °C. The resulting band of adenoviruses was collected and dialyzed four times against 500 ml phosphate buffered saline (PBS) containing 10% glycerol, 2 h each time. The generated Ads were designated as Ad5-IXFlag-IXmRFP1 (containing two types of pIX) and Ad5-IXFlag-IXHis6-IXmRFP1 (containing three types of pIX). Viral particle titers were determined by spectrophotometry at OD260 (Maizel et al., 1968).

Protein electrophoresis and Western blotting

Purified viruses were boled in Laemmli sample buffer for 5 min and separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween-20 followed by primary antibodies incubation (rabbit anti-pIX, 1:1000; mouse anti-Flag, 1:1000; mouse anti-His, 1:1000; rabbit anti-RFP, 1:1000). After washing and re-blocking, the membrane was incubated with corresponding secondary antibodies conjugated to HRP or AP at 1:1000 dilution. The HRP signal was developed with ECL plus Western bloting detection system (GE healthcare, Little Chalfont, UK), detected with BioMax MR scientific imaging film (Kodak, Chalon-sur-Saone, France) and medical film processor SRX-101A (Konica, Tokyo, Japan). AP signal was developed with AP staining solution containing 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium (NBT) according to the manufacturer protocol (Sigma).

ELISA

Solid-phase binding enzyme-linked immunosorbent assays (ELI-SAs) were performed essentially as described previously (Wu et al., 2005). Briefly, 10⁵ VP viruses were subjected to serial dilution (1, 2, 4, 8, 16, 32, 64, 128) in 100 μl of 100 mM carbonate buffer (pH 9.5), and immobilized triplyatlycally in a 96-well plate (Nunc Maxisorp) by overnight incubation at 4 °C. After 4 washes with 0.05% Tween 20 in Tris-buffered saline (TBS-T) and blocking with TBS-T containing 2% bovine serum albumin (BSA), the viruses were probed with primary antibody, and then AP-conjugated secondary antibodies in TBS-T containing 0.5% BSA at room temperature for 2 h, with extensive washes and blocking in between, p-nitrophenyl phosphate (Sigma) was used for color development as described by the manufacturer, and light absorbance (405 nm) was obtained by a microplate reader (PowerWave HT 340, BioTek, Winooski, VT.) after incubation for 90 min.

Fluorescence microscopy

Fluorescence microscopy on purified viral particles was performed as following. 10 μl 50% glycerol/PBS solution containing 5 × 10⁹ VPs of viruses were dropped onto a Fisherfinest™ premium microscope slides (Fisher Scientific, Pittsburgh, PA.) and covered by Fisherband™ #1 cover glass (Fisher Scientific). Fluorescence microscopy was performed with an inverted IX-70 microscope (Olympus, Melville, NY) equipped with a Magnifire digital CCD camera (Optronics, Goleta, CA.).

Quantitative polymerase chain reaction (Q-PCR)

For quantification of adenoviral E4 DNA, TaqMan primers and probes were designed by the Primer Express 1.5 software and synthesized by Sigma Genosys (Woodlands, Texas). The sequences of primers and probe to amplify e4 gene were following: forward primer 5’ - GGAGTGCGCGCAGACACAAC-3’, reverse primer 5’ - ACTACTCGGGGCTTCCT-3’ and probe 6FAM- TGGCATGACACTACGACCAACGATCT - TAMRA. With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a
final volume of 9 μl per reaction containing 1× Universal PCR Master Mix (Applied Biosystems, Foster City, CA.), 100 nM forward primer, 100 nM reverse primer, 100 nM probe and 0.025% BSA. For the assay, known amount of E4 template DNA (10^6, 10^5, 10^4 and 10^2 copies/μl) was amplified to generate a standard curve for quantification of the E4 copy numbers of samples. 1 μl of sample was added to 9 μl of PCR mixture in each reaction capillary. Two no-template-control capillaries received 1 μl of water. All capillaries were then sealed and centrifuged using LC Carousel centrifuge (Roche Molecular Biochemicals, Indianapolis, IN.) to facilitate mixing. PCR was carried out using a LightCycler™ system (Roche Molecular Biochemicals). Thermal cycling conditions were following: 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data was analyzed with LightCycler software 3.

**CAR binding inhibition assay**

293 cells were plated in 48-well plates at a density of 6×10^4 cells per well the day before binding, on which viral particles equivalent to an MOI of 1000 were used for binding assay. Virus were pre-incubated with sCAR (200 μg/ml) protein in DMEM/F12 medium containing 2% FBS at room temperature for 10 min, and then were incubated with cells at 4 °C. After 2 h, cells were washed with cold PBS, harvested by Versene/EDTA. Total DNA was extracted from collected cells using QIAamp DNA Blood mini kit (Qiagen) and subjected to Q-PCR to measure Ad5 E4 copy numbers.

**Genome transfer assay**

293, A549, GH329 or HeLa cells were plated in 12-well plates at a density of 3×10^5 cells per well the day before infection. Viral particles equivalent to an MOI of 100 were used for each infection in DMEM/F12 medium containing 2% FBS at 37 °C. After 2 h, the infected cells were washed by PBS and harvested by Trypsin/EDTA. Total DNA was extracted from collected cells by using QIAamp DNA Blood mini kit (Qiagen) and subjected to Q-PCR to measure Ad5 E4 copy numbers.

**Heat inactivation assay**

Heat inactivation assay was performed essentially as described previously (Dmitriev et al., 2002; Wu et al., 2002, 2005). Briefly, viruses were incubated at 45 °C for 0, 5, 10, 20 or 40 min in either PBS (without Ca^{2+} and Mg^{2+}) or growth medium containing 2% FBS. Then their infective titers were re-determined by standard Tissue Culture Infectious Dose 50 (TCID_{50}) method (AdEasy vector system, Qbiogene, Carlsbad, CA). In brief, 100 μl growth medium containing 2% FBS and 10^4 293 cells were added on two 96-well flat bottom plates the day before infection. Eight serial dilutions of the virus ranging from 10^{-3} to 10^{-10} were made in medium containing 2% FBS and 100 μl of diluted viruses were added into 96-well plates, one row for each dilution. After incubation for 10 days at 37 °C in 5% CO2 humidified incubator, the plates were examined for CPE under microscope. Observable CPE containing wells were counted for each row in order to determine the ratio of positive wells per row in the 96-well plates. Titer was calculated by using KABER statistical method: T (TCID_{50} titer)=10^{9} \times (d^{-0.5})/ml, in which d is the log 10 of the dilution and S is the sum of ratios from the first dilution.

**Statistical analysis**

Statistical analysis was performed with two-tailed unpaired Student's t-tests among groups. P-values < 0.05 were considered statistically significant.

**Growth kinetics**

Growth kinetics of adenovirus was obtained essentially as described previously (Wu et al., 2002). 293 cells were plated in 6-well plates at the density of 3×10^5 cells per well 24 h before infection. The cells were infected with adenoviruses at an MOI of 5 VP/cell in 500 μl growth medium containing 2% FBS. 1.5 ml more growth medium containing 10% FBS was then added into each well after 2 h incubation at 37 °C in 5% CO2 humidified incubator. The infected cells were monitored and harvested with medium at various time point post infection until complete CPE was formed. The collected cells together with the medium were lysed by four cycles of freeze–thaw in dry ice and water bath, and were subjected to centrifugation at 3000 ×g for 30 min at 4 °C for cell debris removal. The total viruses in each well were determined by multiplying TCID_{50} titer with the total volume of the supernatant, and plotted on the diagram as growth curves.

**Immunoelectron microscopy**

Viruses were fixed with 2% paraformaldehyde/PBS at room temperature for 10 min and adhered to 400-mesh nickel grids supported with carbon-coated Formvar film (EMS). After washing with 1% BSA/PBS twice for 10 min each, grids were probed with 1% BSA/PBS diluted primary antibodies (1:200 for goat anti-His6, M2 anti-Flag and rabbit anti-RFP) and incubated at room temperature for 1 h. After 2 cycles of 1% BSA/PBS washes, grids were incubated with 1:40 diluted secondary antibodies (10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit) at room temperature for 30 min. After fixing with 1% glutaraldehyde/PBS for 20 min, grids were subjected to negative staining in 2% uranyl acetate for 12 s and examined under transmission electron microscope in UAB High Resolution Imaging Facility.

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