Published in final edited form as:

J Mol Biol. 2010 January 8; 395(1): 55–78. doi:10.1016/j.jmb.2009.10.034.

***In Vitro* Dynamic Visualization Analysis of Fluorescently Labeled Minor Capsid Protein IX and Core Protein V by Simultaneous Detection**

Hideyo Ugai¹, Minghui Wang¹, Long P. Le², David A. Matthews³, Masato Yamamoto⁴, and David T. Curiel^{1,*}

¹ Division of Human Gene Therapy, Department of Medicine, Obstetrics and Gynecology, Pathology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

² Massachusetts General Hospital, Pathology Service, 55 Fruit St.-GRJ 249, Boston, MA 02114, USA

³ Department of Cellular and Molecular Medicine, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

⁴ Division of Basic and Translational Research, Department of Surgery, University of Minnesota, Minneapolis, MN 55455, USA

Abstract

Oncolytic adenoviruses represent a promising therapeutic medicine for human cancer therapy, but successful translation to human clinical trials requires careful evaluation of these viral characteristics. While the function of the adenovirus proteins have been analyzed in detail, the dynamics of adenovirus infection remain largely unknown due to technological constraints which prevent adequate tracking of the adenovirus particles after infection. Fluorescent labeling of the adenoviral particles is one new strategy designed to directly analyze dynamic processes of viral infection in virus-host cell interactions. We hypothesized that the double labeling technique of adenovirus with fluorescent proteins would allow us to properly analyze intracellular viruses and the fate of viral proteins in live analysis of adenovirus as compared to a single labeling. Thus, we generated a fluorescently labeled adenovirus with both a red fluorescent minor capsid protein IX (pIX-mRFP1) and a green fluorescent minor core protein V (pV-EGFP), resulting in Ad5-IX-mRFP1-E3-V-EGFP. The fluorescent signals for pIX-mRFP1 and pV-EGFP were detected within 10 min in living cells. However, the growth curve analysis of Ad5-IX-mRFP1-E3-V-EGFP showed approximately 150-fold reduced production of the viral progeny at 48 hours post-infection (h.p.i.) as compared to Ad5. Interestingly, pIX-mRFP1 and pV-EGFP were initially localized in the cytoplasm and the nucleolus, respectively, at 18 h.p.i. These proteins were observed in the nucleus during the late stage of infection and the relocalization of the proteins was observed in an adenoviral replication-dependent manner. These results indicate that the simultaneous detection of adenovirus using dual-fluorescent proteins is suitable for real-time analysis, including identification of infected cells, and monitoring viral spread, which will be required for complete evaluation of oncolytic adenoviruses.

*Corresponding author. Mailing address: Division of Human Gene Therapy, Department of Medicine, University of Alabama at Birmingham, 901 19th Street South, BMR2-R502, Birmingham, Alabama 35294 USA. Phone: (205) 934-8627. Fax: (205) 975-7476. curiel@uab.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

adenovirus; fluorescent protein; pV; pIX; imaging

Introduction

Adenoviruses have been developed as vehicles capable of efficiently transferring a transgene into target cells,¹ and genetic engineering has made it possible to tailor adenoviruses as therapeutic agents for human cancer therapy.² In particular, oncolytic adenoviruses, which selectively replicate in tumor cells, but not normal cells, have been designed to specifically kill tumor cells as a direct consequence of viral infection. These oncolytic adenoviruses have been predicted to self-amplify and spread from the initial site of infection throughout the tumor without damaging the surrounding normal tissues. Therefore, virotherapy using oncolytic adenoviruses represents a promising new direction for human cancer therapy.³ Indeed, a variety of oncolytic adenoviruses have been developed,^{2,4,5} and have shown efficacy in many cancer patients.^{6–9} Despite the rapid translation to human clinical trials, little data has been gathered concerning the side-effects or toxicity to normal tissues, effects of pre-existing immunity, and monitoring of viral spread. In fact, it has been demonstrated that the life cycle of oncolytic adenovirus can vary dramatically, and furthermore, the infected cell type is likely to influence the life cycle.^{10–12} Most importantly, the selectivity of viral replication in tumor cells versus normal cells has not been completely evaluated.¹³ Thus, the essential issues concerning the safety and efficacy of oncolytic adenoviruses are poorly understood.

This lack of critical data is due, in part, to the inability to effectively detect and track the infection and spread of adenovirus *in vivo*. Current detection of viruses utilizes the quantification or hybridization of virus DNA and RNA, immunohistochemistry, or the detection of a reporter protein such as luciferase in infected cells. However, these detection methods were developed to indirectly observe viruses in order to isolate the infected sample *in vitro* and *in vivo*. The indirect detection methods are of limited use for detecting replicative adenoviruses in the cycle of virus-host cell interactions. Therefore, a method for dynamically tracking the life cycle of replicative adenoviruses, as well as the expression and localization of the viral proteins, is needed.

In this regard, adenoviruses have previously been genetically engineered to express a monomeric red fluorescent protein (mRFP1), or an enhanced green fluorescent protein (EGFP), in adenoviral minor capsid protein IX (pIX) or core protein V (pV).^{14–17} Protein IX is a structural component of the adenovirus capsid^{18,19} as well as a delayed early viral protein.¹⁸ Since activation of the pIX promoter is only observed by DNA replication of its gene and the transcription of the pIX gene is detected after DNA replication,^{20–23} the transcription of the pIX gene requires replication of the viral genome and the pIX expression appears to be accelerated by the adenoviral genome replication. Therefore, observation of the pIX expression facilitates us to detect infected cells and adenoviral DNA replication. Indeed, the correlation between expression of the fluorescent signal for pIX-mRFP1 fused mRFP1 at the C-terminus of pIX and the replication of the adenoviral genome was observed *in vitro* and *in vivo*.¹⁵ pV, an adenovirus core protein, serves as an alternative site for the fluorescent labeling of adenovirus, allowing examination of core protein expression.¹⁶ Intracellular localization and functional studies of pV performed with the C-terminal fusion of EGFP to the native protein (pV-EGFP) indicate that this labeling does not affect the native cellular localization.^{16,24} Based on these findings, we previously developed a chimeric expression system that allowed incorporation of both genetically labeled pV-EGFP and the native core protein.¹⁶ The fluorescent labeling of adenovirus pV allowed us to efficiently visualize the adenoviral particles as effectively as the capsid labeling of adenovirus.¹⁶ However, the discrimination

between free viral structural proteins and viral assembly associated structural proteins is required in the real-time analysis of intracellular viruses. Therefore, we hypothesize that incorporating fluorescent labels simultaneously on a capsid site and a core site will provide the ability to glean even more critical information regarding the life cycle of adenovirus. First, this double labeling will allow us to visualize the full infection cycle of adenovirus, including the expression of capsid and core proteins. In turn, if each site is labeled with a different fluorophore, fully assembled virus particles could be identified by the change in wavelength as a result of overlapping fluorophores, allowing differentiation of assembled virus particles from unassembled components.

Here, we report that the generation of a fluorescently double labeled human adenovirus type 5 (Ad5), Ad5-IX-mRFP1-E3-V-EGFP, carrying pIX-mRFP1 and pV-EGFP in addition to the native core form of pV. This study revealed that the pIX-mRFP1 and pV-EGFP expression was correlated with viral replication, and that the intracellular movement of pIX-mRFP1 and pV-EGFP was observed during viral replication and at the terminal stage of infection after viral replication. The simultaneous tracking of pIX-mRFP1 and pV-EGFP allowed us to observe the complete cycle of viral replication in infected cells. Thus, the double labeling of adenovirus using two types of fluorescent proteins offers a unique way to more thoroughly monitor adenovirus infection and to study adenovirus biology.

RESULTS

Generation of a fluorescently double labeled adenovirus

To construct plasmid DNA, pAd5-IX-mRFP1-E3-V-EGFP, carrying the Ad5-IX-mRFP1-E3-V-EGFP genome, pShuttle-wt-IX-mRFP1¹⁵ and pAd5-E3-V-EGFP¹⁶ were homologously recombined in *Escherichia coli* strain BJ5183 and Ad5-IX-mRFP1-E3-V-EGFP was generated in 911 cells as described in Materials and Methods section. Figure 1 shows the schematic representation of human adenovirus type 5 (Ad5) and the fluorescently labeled adenoviruses used in this study. Ad5 was previously generated²⁵ using pTG3602²⁶ carrying the Ad5 genome. Ad5-wt-IX-EGFP¹⁴ and Ad5-wt-IX-mRFP1¹⁵ are adenovirus mutants which contain fluorescently labeled pIX with EGFP and mRFP1, respectively. Ad5-dE1-IX-mRFP1²⁷ is an E1-deleted adenovirus carrying the pIX-mRFP1 gene and Ad5-E3-V-EGFP¹⁶ is an E3-deleted adenovirus mutant which contains the pV-EGFP gene in the deleted E3 region. The protein IX (pIX)-modified adenoviruses will only express the fusion protein of pIX in infected cells whereas the pV-modified adenoviruses will express both wild type protein V (pV) and pV-EGFP in infected cells (Fig. 1). The genome sizes of adenoviruses used in this study are shown in Table 1. The genome sizes of all modified adenoviruses were calculated based on the nucleotide sequences of the wild type Ad5 genome (Genbank Accession No. AC_000008), the EGFP gene (Genbank Accession No. U55762), the mRFP1 gene (Genbank Accession No. AF506027).

The biological and physical properties of purified adenoviruses were summarized in Table 1. The viral particle (VP)/plaque forming unit (PFU), fluorescent cell unit (FCU)/PFU, and VP/FCU values of Ad5-IX-mRFP1-E3-V-EGFP were 85, 1, and 101, respectively (Table 1). This result suggests that core modification improve deficiencies of the biological and physical properties of Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1. Ad5-IX-mRFP1-E3-V-EGFP formed an unclear and smaller plaque on A549 cells as compared to Ad5 (data not shown), although the fluorescent signals for pIX-mRFP1 and pV-EGFP were significantly detected in the plaque (Fig. 2). Also, Ad5-IX-mRFP1-E3-V-EGFP with regard to plaque morphology showed a similar characteristic to the other control viruses with a fluorescent label (data not shown).

Alternatively, the VP and PFU titers of Ad5-dE1-IX-mRFP1 were identical with the value previously published by Tang *et al.*²⁷ However, the FCU titer of Ad5-dE1-IX-mRFP1 showed

a dramatically decreased value (Table 1). As a result, the FCU to PFU ratio of Ad5-dE1-IX-mRFP1 was remarkably similar to that of Ad5-IX-mRFP1-E3-V-EGFP and Ad5-E3-V-EGFP (Table 1). This data suggests that the deletion of the E1 region affects the pIX-mRFP1 expression in infected cells.

Ad5-IX-mRFP1-E3-V-EGFP viral particles display and incorporate fluorescently labeled pIX and pV

To characterize protein composition of Ad5-IX-mRFP1-E3-V-EGFP viral particles, 3.0×10^{10} VP of purified adenoviruses were resolved by 10% (w/v) SDS-PAGE and the proteins visualized by GELCODE Blue staining (Fig. 3a). The protein patterns observed in Ad5-IX-mRFP1-E3-V-EGFP appeared to show that modification by fluorescent proteins does not affect relative incorporation of the individual components of the viral particles (Fig. 3a). Detection of the protein bands for pIX-mRFP1 and pV-EGFP in Ad5-IX-mRFP1-E3-V-EGFP was difficult as compared to the control viruses (Fig. 3a). We next assessed the incorporation and display of fluorescently labeled proteins in the adenoviral particles by western blot analysis. Bands for pIX-mRFP1 and pV-EGFP were detected in purified Ad5-IX-mRFP1-E3-V-EGFP as well as the control adenoviruses (Fig. 3b and 3c), indicating that the Ad5-IX-mRFP1-E3-V-EGFP viral particles contained pIX-mRFP1 and pV-EGFP. Signals for pIX-modified proteins were strongly detected in the purified adenoviruses as compared to that for wild type pIX (Fig. 3b) and the proteolytic degradation products of for pIX-modified proteins in purified pIX-modified adenoviruses were also observed by anti-pIX antibody (Fig. 3b, asterisks). Since no detection of wild type pIX gene was analyzed by PCR (Supplementary Figure 1), approximately 15 kDa of protein detected in purified Ad5-IX-mRFP1-E3-V-EGFP was also a proteolytic degradation product of pIX-fusion protein (Fig. 3b, an asterisk). These results revealed that pIX-modified adenoviral particles excessively contain not only the pIX fusion labeled with a fluorescent protein but also the proteolytic degradation products. On the other hand, incorporation of pV-EGFP in the viral particles did not appear to affect wild type pV incorporation as compared to the other viruses (Fig. 3c) and Ad5-IX-mRFP1-E3-V-EGFP contained pV-EGFP along with wild type pV (Fig. 3c, lane 5).

Analysis of viral particle morphology by transmission electron microscopy

Western blot analysis of purified pIX-modified adenoviruses showed that increasing of fluorescently labeled pIXs were detected in the purified adenoviral particles (Fig. 3b). In order to provide a direct demonstration of fluorescently labeled adenoviral particle formation, the adenoviral particles were analyzed by transmission electron microscopy (TEM). The TEM images showed that the size of the Ad5-IX-mRFP1-E3-V-EGFP viral particles was similar to that of Ad5 and the other control viral particles and represented by uniform particles of 70–80 nm in diameter (Fig. 4). Morphology of the Ad5-IX-mRFP1-E3-V-EGFP and Ad5-E3-V-EGFP viral particles was not uniform as compared to that of Ad5 (Fig. 4d and 4e). On the other hand, the Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 viral particles appeared to be sphere-like particles as compared to Ad5 and be coated by those pIX fusions (Fig. 4a–c). In particular, the Ad5-wt-IX-EGFP viral particles contained aggregated proteins which are likely to be pIX-EGFP (Fig. 4b, arrows). The morphologies of the fluorescently labeled adenoviral particles did not indicate empty viral capsids.²⁸ Collectively, the TEM images revealed modification of adenoviral capsid and core proteins causes various morphologies of viral particles.

***In vitro* visualization of Ad5-IX-mRFP1-E3-V-EGFP viral particles**

To further confirm that the Ad5-IX-mRFP1-E3-V-EGFP viral particles contain pIX-mRFP1 and pV-EGFP, we directly visualized the fluorescently labeled viral particles of Ad5-IX-mRFP1-E3-V-EGFP (Fig. 5). Fluorescent signals were detectable for pIX-mRFP1 and pV-EGFP comprised in Ad5-IX-mRFP1-E3-V-EGFP as well as in control adenoviruses.

Furthermore, pIX-mRFP1 and pV-EGFP were adjacently detected in Ad5-IX-mRFP1-E3-V-EGFP viral particles, as expected if they are comprised by the same virus particles (Fig. 5, arrows in panels (f)-(h) for Ad5-IX-mRFP1-E3-V-EGFP). However, fluorescent particulates for Ad5-IX-mRFP1-E3-V-EGFP viral particles were not all of equal size, indicating that Ad5-IX-mRFP1-E3-V-EGFP viral particles form aggregates.

Tracking of the Ad5-IX-mRFP1-E3-V-EGFP viral particles

We previously demonstrated the utility of capsid and core labeling techniques to track the fate of fluorescently labeled pIX and pV during the early stage of infection in cells with Ad5-wt-IX-EGFP, Ad5-wt-IX-mRFP1 and Ad5-E3-V-EGFP.^{14–16} To determine if Ad5-IX-mRFP1-E3-V-EGFP viral particles are tracked in a similar manner to the previously reported viral particles, we monitored the fluorescently labeled adenoviral particles in A549 cells immediately after infection at an MOI of 10,000 VP/cell. At 10 minutes post-infection (m.p.i.) with Ad5-wt-IX-EGFP or Ad5-wt-IX-mRFP1, pIX-EGFP and pIX-mRFP1 could be mainly detected in the cytoplasm and the perinuclear of infected cells, respectively. pV-EGFP was detected in the cytoplasm of Ad5-E3-V-EGFP-infected cells at 10 m.p.i. (Fig. 6a). At 10 m.p.i. with Ad5-IX-mRFP1-E3-V-EGFP, pIX-mRFP1 and pV-EGFP were mainly detected in the cytoplasm and the internalization path was not affected by the dual labeling (Fig. 6a, panels for Ad5-IX-mRFP1-E3-V-EGFP). Fluorescent signal for pIX-mRFP1 was also detected in the nucleus, but the signal was not identical with fluorescent signal for pV-EGFP in the nucleus (Fig. 6a, white and orange arrows in panels for Ad5-IX-mRFP1-E3-V-EGFP). Therefore, pIX-mRFP1 appeared to dissociate from viral particles, suggesting that pIX dissociates from the adenoviral particles during acid-dependent penetration.²⁹

At 10 m.p.i. with Ad5-wt-IX-EGFP or Ad5-wt-IX-mRFP1, the fluorescently labeled pIXs were detected in a punctate staining pattern (Fig. 6a). The punctate pattern is indicative of a vesicular localization as would be expected for viral particles. To detect a significantly punctate staining pattern of Ad5-IX-mRFP1-E3-V-EGFP, we visualized Ad5-IX-mRFP1-E3-V-EGFP in A549 cells immediately after infection at a high MOI (50,000 VP/cell). pIX-mRFP1 and pV-EGFP colocalized in similar punctate vesicles in Ad5-IX-mRFP1-E3-V-EGFP-infected cells (Fig. 6b, arrows), indicating the viral particles co-comprised these fluorescently labeled proteins (Fig. 6b). Taken together, the fluorescent signals for pIX-mRFP1 and pV-EGFP was detected at immediate early stage of infection.

Kinetics of viral replication and fluorescent protein expression of Ad5-IX-mRFP1-E3-V-EGFP

To assess the growth kinetics of Ad5-IX-mRFP1-E3-V-EGFP, we infected Ad5-IX-mRFP1-E3-V-EGFP at an MOI of 10 PFU/cell in A549 cells and tested one-step growth curve kinetics as compared to the control viruses. In a one-step growth curve, the growth kinetics of Ad5-IX-mRFP1-E3-V-EGFP as well as the other modified viruses did not behave identically to Ad5 (Fig. 7a). In the eclipse stage of infection, observed during the first 12 hours after infection in which the viral genome is uncoated from its protective capsid and no infectious virus can be detected inside cells, the low level of viral infectivity observed during the period probably results from adsorbed virus that was not uncoated.³⁰ Therefore, Ad5-IX-mRFP1-E3-V-EGFP appeared to be well uncoated as compared to the other viruses, and we observed the lower level of viral infectivity in all modified adenoviral infected cells during the eclipse period as compared with Ad5 (Fig. 7a). Beginning at 12 h.p.i., the quantity of intracellular infectious virus of Ad5-IX-mRFP1-E3-V-EGFP increased in infected cells during the synthetic phase in which newly infectious viral particles are produced (Fig. 7a). The growth curve of Ad5-IX-mRFP1-E3-V-EGFP as compared to Ad5 indicated a dramatic attenuation of viral growth (Fig. 7a). In addition, the viral growth of parallel infection experiment showed a reduced production of all modified adenoviruses (Fig. 7a). In particular, the yield of the infectious progeny of Ad5-IX-mRFP1-E3-V-EGFP virus by 48 h.p.i. was approximately 150-fold lower as compared to

Ad5 (Fig. 7a). The attenuation of production of pIX-modified adenoviruses was consistent with the property of pIX-modified adenoviruses which was previously reported by Tang *et al.*²⁷ Collectively, the modification of pIX and pV affected the growth property of adenovirus and the production of adenoviral progeny in all modified adenoviruses.

We next measured the cellular uptake of the viral genome and the viral DNA replication in infected cells by quantitative PCR analysis in order to observe whether a dramatic attenuated replication of fluorescently labeled adenoviruses is associated with those DNA replication. The cellular uptake of the Ad5-IX-mRFP1-E3-V-EGFP genome at 1 h.p.i. showed similar value to that of the Ad5 genome (Fig. 7b), and the Ad5-IX-mRFP1-E3-V-EGFP genome replicated similar to the Ad5 genome in infected cells (Fig. 7c). In contrast, the replication of the Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 genomes was different from that of the other adenoviruses, because higher amounts of those genomes were delivered into cells resulting from those higher VP to PFU ratios (Fig. 7b and 7c, and Table 1). Taken together, the modification of pIX and pV of Ad5-IX-mRFP1-E3-V-EGFP did not affect its DNA replication in infected cells.

To further examine the production of virus in infected cells, we next compared the expression levels of pIX-mRFP1 and pV-EGFP in Ad5-IX-mRFP1-E3-V-EGFP-infected cells to control adenoviruses by western blot analysis. The pIX-mRFP1 expression was increased by 24 h.p.i. in cells infected by Ad5-IX-mRFP1-E3-V-EGFP as well as the expression of wild type pIX in Ad5-infected cells (Fig. 7d). The proteolytic degradation products of pIX-mRFP1 were also detected in Ad5-IX-mRFP1-E3-V-EGFP-infected cells as well as Ad5-wt-IX-EGFP- and Ad5-wt-IX-mRFP1-infected cells (Fig. 7d). Overexpression of fluorescently labeled pIXs was also observed in infected cells as compared to Ad5- and Ad5-E3-V-EGFP-infected cells (Fig. 7d). Since the promoter for the pIX gene is intact in all replication-competent adenoviruses used in this study, the gene expression of pIX should be similarly activated in infected cells. Therefore, these data suggest that modification of pIX by the fluorescent proteins may enhance the pIX stability.^{31,32} Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 infection induced the expression of fluorescently labeled pIXs as well as that of pV at an earlier time point (12 h.p.i.), since the replication of the genomes started by 6 h.p.i. in infected cells due to delivery of higher amounts of those genomes into cells (Fig. 7b and 7d).

The expression of wild type pV was observed by 24 h.p.i. in cells infected by Ad5, Ad5-E3-V-EGFP or Ad5-IX-mRFP1-E3-V-EGFP (Fig. 7e). However, the pV-EGFP expression was detected at an earlier time point as compared with the wild type pV expression in Ad5-E3-V-EGFP- and Ad5-IX-mRFP1-E3-V-EGFP-infected cells (Fig. 7e). Western blot analysis of the pV expression observed by 24 h.p.i. in Ad5-infected A549 cells was reproductive (data not shown). Importantly, the pIX-mRFP1 and pV-EGFP expressions were elevated by the DNA replication-dependent manner of Ad5-IX-mRFP1-E3-V-EGFP as observed in the other fluorescently labeled viral infections.

We also analyzed the growth property of Ad5-IX-mRFP1-E3-V-EGFP by fluorescent microscopy. Particularly, pIX is a delayed early viral protein¹⁸ and the pIX transcription is activated by its DNA replication.^{20–23} In addition, the E3 promoter for the pV-EGFP gene is regulated by E1 protein.^{33–35} We first confirmed adenoviral infectivity in A549 cells by using Ad5-dE1-CMV-EGFP and observed EGFP signal in most of A549 cells at 24 h.p.i. (Supplementary Figure 2a). The pIX-mRFP1 expression was dramatically increased in A549 cells infected by a replication-competent adenovirus with pIX-mRFP1, Ad5-wt-IX-mRFP1, as compared to that infected by a replication-deficient adenovirus with pIX-mRFP1, Ad5-dE1-IX-mRFP1 (Fig. 8a). The pIX-mRFP1 expression mediated by Ad5-dE1-IX-mRFP1 was also detected in some infected cells at 36 h.p.i. (Fig. 8a), resulting from the DNA replication of E1-deleted adenoviruses without contamination of replication-competent adenovirus

(Supplementary Figure 2b and 2c). This data was identical with previous reports of the DNA replication of E1-deleted adenoviruses slowly occurred in cancer cells.^{36–38} Thus, the pIX expression was accelerated by the adenoviral DNA replication (Fig. 8a). The fluorescent signals for pV-EGFP and pIX-mRFP1 were detectable at 12 and 24 h.p.i., respectively, in a proportion of Ad5-IX-mRFP1-E3-V-EGFP-infected cells (Fig. 8b, arrows). Of note, the fluorescent signals for pIX-mRFP1 and pV-EGFP increased over time in infected cells (Fig. 8b), suggesting the expression of fluorescent proteins was elevated in a viral DNA replication-dependent manner. Moreover, Ad5-IX-mRFP1-E3-V-EGFP infection induced cytopathic effect (CPE) similar to Ad5 (Fig. 8c, panels for phase contrast). The time-frame for the expressions of pIX-EGFP, pIX-mRFP1 and pV-EGFP was similar following infection of cells with Ad5-wt-IX-EGFP, Ad5-wt-IX-mRFP1 or Ad5-E3-V-EGFP, respectively (Fig. 8c). These results of fluorescent microscopic analysis are consistent with the results obtained from western blot analysis of the pIX-mRFP1 and pV-EGFP expression (Figs. 7 and 8b). Therefore, these data suggest that the expression of fluorescent proteins was elevated by the DNA replication of Ad5-IX-mRFP1-E3-V-EGFP.

Localization of fluorescently labeled pIX and pV

Adenovirus pV localizes in the nucleolus and nucleus when it is transiently expressed using a plasmid carrying the pV gene.³⁹ In addition, previous studies suggested that pIX is normally localized in the nucleus at the intermediate stage of infection (14 to 16 h.p.i.). On the other hand, pIX-GFP transiently expressed in the cytoplasm of cells infected with an E1-deleted adenovirus does not appear to preferentially accumulate into the nucleus.¹⁷ Furthermore, the fate of pIX and pV is not well understood in cells infected by a replication-competent adenovirus. Therefore, we simultaneously analyzed the fate of pIX and pV by fluorescent labeling technique. The pIX-EGFP and pIX-mRFP1 were preferentially localized in the cytoplasm of cells infected with recombinant adenoviruses expressing one fluorescent label or both fluorescent labels at 18 h.p.i. (Fig. 9). In particular, pIX-EGFP tended to aggregate in the cytoplasm of infected cells (Fig. 8b and 9c, arrows), although EGFP did not aggregate in cells.¹⁴ On the other hand, pV-EGFP was mainly localized in the nucleoli of infected cells (Fig. 8h, 8k and 8m) and the localization of pV-EGFP observed at 18 h.p.i. in infected cells was identical with previous reports published by Le *et al.* and Matthews *et al.*^{16,39} Also, pV-EGFP appeared to be concentrated in the nucleus (Fig. 9h, 9k and 9m, arrowhead) as described in the previous report by Matthews *et al.*³⁹ Interestingly, at 18 h.p.i. with Ad5-IX-mRFP1-E3-V-EGFP, the pIX-mRFP1 expression was not obviously detected in most infected cells which were expressing pV-EGFP (Fig. 9l). This is likely due to the expression of pV-EGFP at an earlier time point as observed in western blot analysis (Fig. 7e). These data indicate that the localization of pIX was exclusively restricted in the cytoplasm at 18 h.p.i., while the pV-EGFP protein accumulated in the nucleus of infected cells (arrowhead in Fig. 9h, 9k and 9m).

Relocalization of pIX-mRFP1 and pV-EGFP during the late stage of infection

Adenoviral progeny is produced in the nucleus of infected cells.^{40–43} In addition, adenovirus is released from infected cells by 36 h.p.i., and sufficient amounts of infectious adenovirus is yielded in the culture medium after 96 h.p.i. at a concentration comparable to that produced in infected cells.⁴⁴ Accordingly, the translocation of pIX and pV to the nucleus is required for viral assembly. To observe the translocation of pIX-mRFP1 and pV-EGFP into the nucleus for viral assembly and release, we simultaneously tracked pIX-mRFP1 and pV-EGFP during the late stage of infection with Ad5-IX-mRFP1-E3-V-EGFP. While the pV-EGFP expression was enhanced in the nucleus by 36 h.p.i., pIX-mRFP1 also began translocating to the nucleus where it appears to surround pV-EGFP (Fig. 10a). Moreover, pIX-mRFP1 and pV-EGFP co-localized in the nucleus of infected cells at 36 h.p.i. when adenoviral progeny were sufficiently produced in infected cells (Figs. 7a and 10a). Since the yield of infectious adenovirus reached a plateau at 36 h.p.i. (Fig. 7a), we observed the relocalization of pIX-mRFP1 and pV-EGFP accompanied

the production of infectious viruses. By 72 h.p.i., pIX-mRFP1 which revealed speckled distribution in the nucleus appeared to colocalize adjacent to pV-EGFP rich centers (Fig. 10a). This speckled distribution of pIX labeled with a fluorescent protein was also detected in the nucleus of Ad5-wt-IX-EGFP- and Ad5-wt-IX-mRFP1-infected cells (Fig. 10b). However, the fluorescently labeled adenoviral particles of Ad5-wt-IX-mRFP1 and Ad5-wt-IX-EGFP released from the nucleus to the cytoplasm following viral assembly were not detectable, because those infected cells shrunk during the late stage of infection. Interestingly, we observed that pV-EGFP was detected in the nuclear membrane of infected cells and in the cytoplasm at 144 h.p.i. (Fig. 10a, enlarged areas (iii) and (iv)). The punctate structures of pV-EGFP were also detected in the cytoplasm in Ad5-E3-V-EGFP-infected cells during the terminal late stage of infection (Fig. 10c). No co-localization of pIX-mRFP1 with pV-EGFP in the cytoplasm at 144 h.p.i. was observed (Fig. 10a, (i)-(iv)). Therefore, the fluorescent signal for pV-EGFP observed in the cytoplasm of Ad5-E3-V-EGFP- and Ad5-IX-mRFP1-E3-V-EGFP-infected cells did not appear to be associated with the viral particles. Importantly, these results indicate that pIX-mRFP1 and pV-EGFP relocated during viral replication for viral assembly. Also, the localization and fate of pIX and pV were determined by fluorescent labeling technique under replicated condition.

DISCUSSION

Fluorescently labeled viruses are of use to directly analyze dynamic processes of virus-host cell interactions and the life cycle of a replicative virus and viral proteins. In this regard, fluorescence-labeling techniques have been successfully applied to adenovirus structural proteins.^{14–16,45,46} In fact, our previous studies showed that adenoviral minor core protein V and minor capsid protein IX are suitable sites to incorporate fluorescent proteins which allow successful detection of those modified adenoviruses.^{14–16} However, the characteristics of fluorescently labeled adenoviruses are not well understood due to the difference between intracellular viruses and free proteins labeled with a fluorescent protein. In addition, the discrimination between free viral structural proteins and viral assembly associated structural proteins is required in the real-time analysis of intracellular viruses. Moreover, the discrimination between infected and uninfected cells is also required for reliable *in vivo* and *in vitro* studies. Therefore, we proposed a differential labeling approach using two kinds of viral structural proteins in live analysis of adenovirus to accurately detect intracellular viruses in infected cells. We report herein the capacity of a fluorescently double labeled adenovirus, Ad5-IX-mRFP1-E3-V-EGFP, with a red fluorescent minor capsid protein IX and a green fluorescent minor core protein V, to provide analysis of virus-host cell interactions involved in the production of viral progeny. Additionally, the direct detection method using Ad5-IX-mRFP1-E3-V-EGFP is useful for detecting replicative adenovirus and for dynamically tracking the life cycle of replicative adenoviruses as well as the expression and localization of the viral proteins.

The FCU titer was determined by counting of the number of cells expressing fluorescent protein visualized by fluorescence microscopy after 48 h.p.i. One PFU is defined as follows: a single viral particle infecting a single cell in a monolayer will give rise to a plaque through multiple rounds of infection.⁴⁷ Thus, PFU is a reliable measure of the number of infectious viral particles in the virus preparation, and allows us to determine whether the FCU titer of the fluorescently labeled adenoviruses is equivalent to the PFU titer. The results of the FCU and PFU assays showed that most cells expressing the fluorescently labeled core and capsid proteins after Ad5-IX-mRFP1-E3-V-EGFP or Ad5-E3-V-EGFP infections produced corresponding infectious viral progenies. Additionally, Ad5-IX-mRFP1-E3-V-EGFP retains the ability to properly trace protein expression correlated with viral replication and cells producing viral progeny as compared to Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 due to a defect in the ability to produce infectious viral progeny.

According to the method of determining viral particle titer reported by Maizel *et al.*,⁴⁸ the titer is calculated based on protein absorbance of the capsid and core protein of purified the adenoviral particles at 260 nm under denaturing conditions using SDS (an OD₂₆₀ of 1.0 corresponds to 0.28 mg/ml protein in SDS-buffer). In this way, particle titer of adenovirus is calculated to be 1.1×10^{12} particles/ml per OD₂₆₀ unit. Since our modified capsid and core proteins have increased molecular mass, this mass increase would affect the calculated viral particle titer. Therefore, we did not use VP titer as calculated by the method of Maizel *et al.*⁴⁸ for analysis of proteins.

We detected strong signals for fluorescently labeled pIXs by western blot analysis compared to the signal for wild type pIX (Fig. 3b). In particular, we directly observed aggregation of viral proteins on the viral particles of Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 (Fig. 4). Protein IX forms a trimer at its N-terminus, and recent reports show that the C-terminal domains of pIX assemble into an antiparallel structure on the facets of the capsid.^{49,50} Free pIX may interact with pIX incorporated in the adenoviral particles. In addition, EGFP forms a weak dimer,⁵¹ and aggregation of pIX-EGFP was observed in Ad5-wt-IX-EGFP-infected cells (Fig. 10b). Therefore, EGFP may also affect aggregation of pIX thorough EGFP domain of the pIX fusion protein. Taken together, increased capsid-association of pIX-modified protein and proteolytic degradation products in the viral particles appeared to cause non-infectious viral particles.

Adenovirus binds the coxsackie B virus and adenovirus receptor (CAR) within 5 min²⁹ and the fluorescent signal for capsid-incorporated pIX-GFP was detected in the cytosol of infected cells 10 min post-infection.^{17,29} We also confirmed a rapid trafficking of pIX-mRFP1 and pV-EGFP by microscopic analysis of cells at 10 m.p.i. without synchronization of the cell entry process at 4°C (Fig. 6). Greber *et al.* reported that pIX dissociates from the incoming viral particles during acid-dependent penetration²⁹ and localization of pIX associated with the adenoviral particles appeared to be different from that of the dissociated pIX. Our studies demonstrated that the fluorescent signals for pIX-mRFP1 and pV-EGFP at immediate early stage of infection showed different localization patterns (Fig. 6a). On the other hand, pV is a minor core protein and is speculated to function as a bridge between the core and the capsid proteins.^{52–54} Each adenoviral virion contains 157 copies of pV, non-specifically associated with the double stranded viral genomic DNA.^{55–58} Therefore, pV does not appear to be released from the viral particles in the penetration process and localization of pV-EGFP seems to be different from that of pIX-mRFP1 dissociated from the viral particles (Fig. 6a). Moreover, the co-localization of the fluorescent signals for pIX-mRFP1 and pV-EGFP following infection with Ad5-IX-mRFP1-E3-V-EGFP at high MOI suggests that the viral particles comprise both proteins (Fig. 6b). High concentrations of the adenoviral proteins induces initial infection through pinocytosis rather than the endocytosis that follows binding the coxsackievirus and adenovirus receptor (CAR).⁵⁹ Therefore, the punctate staining pattern of the fluorescent signals for fluorescently labeled proteins observed in the cytoplasm at high MOI was likely to be intact viral particles entering via pinocytosis (Fig. 6b). As a result, the localization of fluorescent signals for pIX and pV fusion proteins detected at high MOI was different from that detected at low MOI (Fig. 6).

The modification of minor capsid and core proteins resulted in decreased infectivity of adenovirus due to the interference in native pIX and pV functions (Fig. 7a). The yield of Ad5-IX-mRFP1-E3-V-EGFP was attenuated approximately 150-fold by pIX-mRFP1 and pV-EGFP proteins at 48 h.p.i. (Fig. 7a). Of note, this level of alternation is much less than the other adenoviral mutants such as VA mutants and temperature sensitive mutants.^{60–62} However, the modification of pIX and pV in Ad5-IX-mRFP1-E3-V-EGFP did not affect its DNA replication due to delivery of similar amount of the adenoviral genome into cells (Fig. 7c). On the other hand, we observed the expression of pV-EGFP at an early time point compared to native pV

expression (Fig. 7e). This result suggests that the E3 promoter regulates the pV-EGFP gene expression inserted in the deleted E3 region^{33,63–65} via E1A/E1B^{33–35} rather than the major late promoter. Therefore, the expression of pV-EGFP at an earlier time point decreased viral replication, because the deletion of the E3 region and the insertion of a transgene in the deleted E3 region does not affect viral replication *per se*.⁶⁶ In addition, the pIX modification dramatically increased its expression (Fig. 7d), suggesting that the pIX-fluorescent protein fusions showed increased protein stability rather than the stability of mRNA for the fusion gene.^{31,32} The overexpression of the modified pIX did not affect viral replication during 12 to 24 h.p.i. in Ad5-wt-IX-EGFP- and Ad5-wt-IX-mRFP1-infected cells (Fig. 7a), but inhibited the production of viral progeny at 48 h.p.i. (Fig. 7a). Taken together, the combination of pV-EGFP and pIX-mRFP1 caused more impact in viral replication of Ad5-IX-mRFP1-E3-V-EGFP as compared to the other viruses (Fig. 7a).

Although Ad5-IX-mRFP1-E3-V-EGFP acquired some defects due to its modifications, the cytotoxicity induced by Ad5-IX-mRFP1-E3-V-EGFP infection was similar to that induced by Ad5 (Fig. 8b). The cytotoxicity induced by adenoviral infection is a secondary effect of its infection resulting from overexpression of late viral proteins in infected cells, and is observed after viral replication.⁶⁷ Development of a fluorescently labeled control adenovirus with similar cytotoxicity to Ad5 is important for evaluating the therapeutic potency of oncolytic adenoviruses via *in vivo* imaging. Ad5-IX-mRFP1-E3-V-EGFP could be utilized for the monitoring of adenovirus infection *in vivo* and as a control virus to compare tumor killing activity of oncolytic adenoviruses with or without an imaging modality. Thus, the characteristics of Ad5-IX-mRFP1-E3-V-EGFP make it suitable for *in vivo* analysis.

The expression of pV-EGFP in Ad5-E3-V-EGFP-infected cells correlated with viral DNA replication (Fig. 7c and 7d), and Ad5-E3-V-EGFP infection induced cytotoxicity similar to Ad5 and Ad5-IX-mRFP1-E3-V-EGFP infection (Fig. 8b and 8c). However, translocation of pV-EGFP from the nucleoli to the nucleoplasm was observed before the pIX-mRFP1 expression, and translocation of pIX-mRFP1 from cytoplasm to nucleoplasm in Ad5-IX-mRFP1-E3-V-EGFP-infected cells (Fig. 9). Therefore, Ad5-E3-V-EGFP is not suitable to track the events associated with adenovirus assembly. In contrast, Ad5-IX-mRFP1-E3-V-EGFP is useful for simultaneous analysis of the fate of pIX and pV associated with adenovirus assembly in infected cells and the intracellular movements of fluorescently labeled viral particles, as compared with the other fluorescently labeled adenoviruses. Our data show change in localization of fluorescently labeled pIX during infection (Fig. 10). As a result, fluorescently labeled pIX which detected in the nucleus during infection is involved in adenovirus assembly. Furthermore, our data indicate that pV-EGFP is involved in virus-induced reorganization which formed specific nuclear structures in which pIX-mRFP1 accumulates during the late stage of infection (Fig. 10a). In addition, the fluorescent flecks for pV-EGFP were observed in the cytoplasm of cells infected by Ad5-IX-mRFP1-E3-V-EGFP as well as Ad5-E3-V-EGFP at the terminal stage of viral infection (Fig. 10a and 10c). Thus, fluorescent labeling techniques of replication-competent adenoviruses allowed us to accurately observe unique aspects of viral replication and the fate of viral proteins which are not detected in cells infected by a replication-deficient adenovirus or transfected by a plasmid carrying an adenovirus gene.

The adenovirus genome encodes a gene for a cysteine protease which recognizes the known consensus motifs, (M,I,L)XGX/G and (M,I,L)XGG/X contained in precursor proteins where X is any residue, and cleaves them at the site of “/”.^{68,69} In particular, the adenoviral protease plays a role in maturation of adenoviral proteins by cleaving precursors of IIIa, VI, VII, VIII, mu, and terminal protein.^{68–70} Also, the adenoviral protease preferentially cleaves II, V, and 100K *in vitro* as well as the above precursor proteins and putative cleavage motifs in the protein sequences for V and 100K have been reported.⁶⁹ We observed the proteolytic degradation products of fluorescently labeled pIXs in purified adenoviruses (Fig. 3) and in lysates of

fluorescently labeled adenovirus-infected cells (Fig. 7d). We found that the protein sequences for EGFP and mRFP1 contain a complete consensus motif and the putative cleavage motifs for the adenoviral protease. Since no degradation of fluorescent proteins by cellular proteins is reported, the adenoviral protease could be responsible for degradation of fluorescently labeled pIXs.

In summary, we demonstrate that the characteristics of Ad5-IX-mRFP1-E3-V-EGFP observed in cell culture support the monitoring of adenovirus infectivity that accompanies the production of infectious viral progeny. Double labeling of adenoviruses using fluorescent proteins complemented defects of the single capsid or core labeling technique. The double labeling technique could allow observation of the interaction between viral and cellular proteins associated with viral infection. Moreover, the double labeling of a replication-competent adenovirus could clarify not only the dynamic mechanisms of viral infection but also the molecular mechanisms involving viral and cellular proteins as observed in cells transiently expressing a viral protein. However, this study shows two future extensions of this fluorescent labeling technique of the adenoviral particles. One is to establish a method to normalize physical properties of adenovirus particles with a high VP to PFU ratio instead of using VP titer. Methods may include calculation of the adenoviral genome titer based on the observation that a solution of 50 µg/ml of purified DNA has an absorbance of 1.0 at 260 nm. The second is to make novel fluorescent proteins which are resistant to the adenoviral protease by site-direct mutagenesis for fluorescent labeling of adenovirus. These future studies would allow the efficient tracking of adenovirus in the basic study of adenovirus biology, pathogenesis, vector design and *in vivo* applications.

Materials and Methods

Cells

Human embryonic kidney 293 cells (293 cells; ATCC CRL 1573; ATCC, Rockville, MD, USA), human embryonic retinoblast 911 cells,⁷¹ 293F28 cells⁷² which is a 293 cell line stably expressing the fiber protein of human adenovirus type 5 (Ad5) and human lung epithelial A549 cells (ATCC CCL 185) were cultured in Dulbecco's modification of Eagle's-Ham's F12 50:50 medium (Mediatech, Inc; Herndon, VA) that contained 10% fetal bovine serum (FBS; Hyclone; Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin. The cells were maintained as subconfluent adherent cultures in an atmosphere of 5% CO₂ in air at 37 °C. The adenovirus infected cells were cultured in the same medium containing 2% FBS.

Viral generation, propagation and purification

Ad5 is human adenovirus type 5 previously generated using pTG3602.^{25,26} Ad5-wt-IX-EGFP¹⁴ and Ad5-wt-IX-mRFP1¹⁵ are adenovirus mutants which express fluorescently labeled minor capsid protein IX with an enhanced green fluorescent protein (EGFP) and a monomeric red fluorescent protein (mRFP1), respectively. Ad5-dE1-IX-mRFP1 is an E1-deleted adenovirus mutant which express fluorescently labeled minor capsid protein IX with mRFP1.²⁷ Ad5-E3-V-EGFP is an E3-deleted adenovirus mutant which express core protein V with an enhanced green fluorescent protein (pV-EGFP) in the E3 region.¹⁶

To construct pAd5-IX-mRFP1-E3-V-EGFP carrying the IX-mRFP1 gene in the native region and the V-EGFP gene in the deleted E3 region, pShuttle-wt-IX-mRFP1¹⁵ and pAd5-E3-V-EGFP¹⁶ were homologously recombined in *E. coli* strain BJ5183. To generate Ad5-IX-mRFP1-E3-V-EGFP, pAd5-IX-mRFP1-E3-V-EGFP was digested with the PacI restriction enzyme, followed by transfection using UniFECTOR reagent (B-Bridge International Inc., Sunnyvale, CA) into 911 cells. Ad5, Ad5-dE1-IX-mRFP1, Ad5-wt-IX-EGFP and Ad5-wt-IX-

mRFP1 were propagated in 293 cells, and Ad5-E3-V-EGFP and Ad5-IX-mRFP1-E3-V-EGFP were propagated in 293F28 cells.

Virus purification and titration

All adenoviruses were purified by two rounds of ultracentrifugation on a CsCl gradient as described elsewhere.⁷³ The particle titer (VP/ml) was determined by A260 absorbance of purified particles and assuming that 1.1×10^{12} VP/ml has an absorbance of 1.0 at 260 nm.⁴⁸ In brief, the particle titer is calculated based on protein amount of the purified adenovirus observed at absorbance 260 nm under denaturing conditions using SDS (an OD₂₆₀ of 1.0 corresponds to 0.28 mg/ml protein in SDS-buffer). A value of 1.77×10^8 daltons for the molecular mass of the viral particle is obtained by dividing 2.3×10^7 daltons (the molecular mass of adenovirus DNA) by 0.13, because 13% of an adenoviral particle is DNA. Next, 1.54×10^8 daltons, the molecular mass of the adenoviral proteins which composed the viral particle, is calculated by subtracting 2.3×10^7 daltons (the molecular mass of the adenovirus DNA) from 1.77×10^8 daltons (the molecular mass of the viral particle). Finally, 1.1×10^{12} particles/ml per OD₂₆₀ unit is obtained by dividing 2.8×10^{-4} g/ml protein by the value of 1.54×10^8 daltons, the molecular mass of the adenoviral proteins, and multiplied Avogadro's number, 6.02×10^{23} . Fluorescent focus assay followed by plaque formation assay were performed to determine the fluorescent cell units titer (FCU/ml) and the infectious titer (PFU/ml) of fluorescently labeled adenoviruses and A549 and 911 cells were used for replication-competent and deficient adenoviruses, respectively, in these assays. In brief, adenovirus was serially diluted with medium contained 2% FBS and infected cells in 6-well plate. After 1 hour post-infection (h.p.i.), medium was removed and 3 ml of DMEM/F12 that contained 2% FBS and 0.75% agar (MBA, Rockland, ME, USA) were laid over on infected cells. After 48 h.p.i., the number of cells expressing the fluorescent protein was counted using fluorescent microscope to determine the FCU titer. The number of cells expressing fluorescent protein was counted using fluorescent microscope, and the titers of viral particles with ability to express fluorescent protein were calculated as fluorescent cell units (FCU) per ml. Subsequently, the same plates used to measure FCU titer were still incubated for 14 days and the plaques generated by adenovirus were counted to determine the infectious titers of adenoviruses.

Transmission electron microscopy

1.0×10^9 VP of purified adenovirus was transferred in a 1.5-ml tube and vortexed for 30 sec to separate each viral particle. Next, adenovirus was fixed with an equal volume (approximately 10 μ l) of fixative buffer (1.25% of glutaraldehyde, 2% paraform-aldehyde in 30 mM HEPES buffer [pH 7.4]) and stored at room temperature for 30 min. 10 μ l of the fixed adenoviral particles were dropped on parafilm and a formvar coated nickel grid (400-mesh, Electron Microscopy Sciences, Hatfield, PA) was placed on the viral solution for 5 min. The nickel grid was washed with 100 μ l of PBS on parafilm, three times at room temperature for 5 min. The adenoviral particles on a grid were stained with 1% (w/v) uranyl acetate at room temperature for 1 min and subsequently the grids were air dried. Transmission electron microscopy (TEM) for the adenoviral particles was performed with FEI-Tecnaï T12 TWIN 120kV and FEI-Tecnaï F20 200kV at the High Resolution Imaging Facility in University of Alabama at Birmingham.

In vitro visualization and tracking of fluorescently labeled adenoviruses

For *in vitro* visualization of viral particles which incorporate fluorescently labeled pIX and/or pV proteins, 1.0×10^9 VP of purified adenoviruses were dropped on a glass slide. The samples were mounted with a cover glass and sealed for fluorescent microscopy. Fluorescent signals were detected by a fluorescent microscope.¹⁴

For the tracking of fluorescently labeled adenovirus at the early stage of viral infection in A549 cells, A549 cells were infected by fluorescently labeled adenoviruses at multiplicities of

infection (MOI) of 10,000 and 50,000 VP/cell with growth medium in a glass-bottom dish (FluoroDish, World Precision Instruments, Inc., Sarasota, FL) and the infected cells were incubated at 37 °C for 10 min. The growth medium were removed and washed three times with phosphate-buffered saline (PBS [pH 7.4]) and then fixed with PBS (pH 7.4) containing 10% formalin for 10 min. After another three washes with PBS, Hoechst 33342 (Sigma-Aldrich, Saint Louis, Mo) staining was performed for 10 min. The fluorescent signals were detected by fluorescent microscopy.

One-step growth curve analysis of adenoviruses

The number of A549 cells, grown to 80% confluency in 6-cm dishes, was counted before viral infection to determine a multiplicity of infection (MOI). A549 cells were infected with adenoviruses at an MOI of 10 PFU/cell and the infected cells were maintained in 3 ml of medium in each dish. The infected cells and the culture medium were harvested with a scraper at various times post-infection and the cell suspension was centrifuged at $1,000 \times g$ for 5 min at 4 °C to separate the cells from the culture medium. The cell pellet was resuspended in 1 ml of PBS (pH 7.4) containing 10% glycerol. The cell suspension was disrupted by three times of freeze and thaw cycles, and the lysate was centrifuged at $15,000 \times g$ for 5 min at 4 °C to remove cell debris. The supernatant was used as the crude virus extracted from infected cells. The crude adenovirus was stored at -80 °C prior to the titration.

Quantitative real-time PCR analysis

A549 cells were grown to 80% confluency in 12-well plate and approximately 2×10^5 cells were contained in a well. Adenovirus infected A549 cells at an MOI of 10 PFU/cell and the infected cells were maintained in 1 ml of medium containing 2% FBS. The infected cells were harvested at various times post-infection by using a cell lifter and centrifuged at $1,000 \times g$ for 5 min to remove the medium. The infected cells were resuspended with 1 ml of PBS (pH 7.4) and centrifuged at $1,000 \times g$ for 5 min to remove PBS (pH 7.4). Total cell DNA containing the adenoviral genome was extracted from the infected cells by a method described previously⁷⁴ and was stored at -80 °C prior to quantitative PCR (qPCR) analysis. Oligonucleotides corresponding to the sense strand of the adenoviral E4 region (5'-TGACACGCATACTCGGAGCTA-3': corresponding to nucleotide [nt] positions 34,881-34901; Genbank accession No. AY339865), the antisense strand of the E4 region (5'-TTTGAGCAGCACCTTGCATT-3': 34972-34952 nt positions), and a TaqMan probe (5'-CGCCGCCCATGCAACAAGCTT-3': 34946-34926 nt positions) were synthesized and used as PCR primers and TaqMan probe for qPCR. The qPCR condition was as follows: 35 cycles of denaturation (94°C, 20 sec), annealing (55°C, 20 sec), and extension (72°C, 30 sec). The Ad5 genome was used to generate a standard curve for the adenoviral E4 DNA copy number. Each reaction for qPCR was performed with 50 ng of total DNA and control sample without template.

Western blot and gel-staining analyses

3.0×10^{10} VP of purified adenoviruses were run on a 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with GELCODE Blue reagent (Pierce Biotechnology Inc., Rockford IL). Rabbit polyclonal anti-pV antibodies and rabbit polyclonal anti-pIX antibodies were a kind gift of Dr. D. A. Matthews (Department of Cellular and Molecular Medicine, School of Medical Sciences, University of Bristol, UK) and from Dr. I. Dmitriev (Gene Therapy Center, University of Alabama at Birmingham), respectively. Secondary HRP-conjugated donkey antibodies against rabbit IgG were purchased from Amersham Biosciences Corp. (Piscataway, NJ). Western blot analysis was carried out as described elsewhere.⁴⁴ Signals were visualized with an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Biosciences Corp.). To analyze the

incorporation of pIX and pV in the viral particles, 3.0×10^{10} or 1.0×10^{10} VP of purified adenoviruses were separated by 12% (w/v) and 10% (w/v) SDS-PAGE, respectively. Proteins that had been separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany) for pIX detection at 300 mA for 30 min and a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics GmbH, Mannheim, Germany) for pV detection at 300 mA for 60 min. Western blot analyses were performed by using anti-pIX and pV.

The crude protein to analyze viral protein expression in infected cells was prepared as follows. After counting the number of A549 cells that had been grown to 80% confluence in 6-cm dishes, cells were infected with adenoviruses at an MOI of 10 PFU/cell. The infected cells were maintained in 3 ml of medium in each dish. The infected cells and the culture medium were harvested with a scraper at various times post-infection and the cell suspension was centrifuged at $1,000 \times g$ for 5 min at 4 °C. The cell pellet was resuspended in 5 ml of PBS (pH 7.4) and the cell suspension was centrifuged at $5,000 \times g$ for 1 min at 4 °C. The cell pellet was resuspended with 100 μ l of RIPA buffer and the lysate was centrifuged at $15,000 \times g$ for 5 min at 4 °C to remove cell debris. The supernatant was used as the crude protein extracted from infected cells. Protein concentration of total protein extracted from adenoviral infected cells was determined using the D_C protein assay kit (Bio-Rad laboratories Inc., Hercules, CA) according to the manufacturer's instructions. The crude protein was stored at -80 °C prior to western blot analysis. 10 μ g of total proteins extracted from virus-infected cells were separated by 12% (w/v) SDS-PAGE for pIX detection and 10% (w/v) SDS-PAGE for pV detection. SDS-PAGE and western blot analysis were performed under the same conditions.

Expression and localization of pIX and pV labeled with fluorescent protein in infected cells

The number of A549 cells, grown to 80% confluency in 6-cm dishes, was counted before viral infection to determine the MOI. A549 cells were infected with adenoviruses at an MOI of 10 PFU/cell and the infected cells were maintained in 3 ml of medium in each dish. We incubated the infected cells at 37 °C and observed the expression of viral proteins fused by fluorescent proteins at various time points post-infection in infected cells. The signals for the fluorescent proteins were detected at a low magnification ($\times 200$) by fluorescent microscope.

A549 cells were cultured in 6-well plate containing a cover-glass. The number of A549 cells was counted before viral infection to determine the MOI. A549 cells were infected with fluorescently labeled adenoviruses at an MOI 10 PFU/cell and the infected cells were incubated at 37 °C for various times. The growth medium was removed and the infected cells were washed three times with PBS and then fixed with PBS containing 10% formalin for 10 min. DNA staining was performed using Hoechst 33342 for 10 min before mounting and sealing. To observe the localization of pIX-EGFP, pIX-mRFP1 and pV-EGFP in infected cells, we performed fluorescent microscopic analysis at a high magnification ($\times 1,000$).

Fluorescent microscope analysis

Fluorescent microscopy was performed with an inverted IX-70 microscope (Olympus, Melville, NY) equipped with a Magnifire digital CCD camera (Optronics, Goleta, CA) and a DP71 digital camera (Olympus). The images of fluorescent signals for EGFP and/or mRFP1 and Hoechst 33342 were merged using Adobe Photoshop CS (San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Roger Y. Tsien (University of California at San Diego) for providing the mRFP1 construct and Dr. Igor P. Dmitriev (University of Alabama at Birmingham) for providing a rabbit polyclonal pIX antibody. We also thank Drs. Anton V. Borovjagin, Erin E. Thacker, George C Dobbins, Joel N. Glasgow, Matthew S. Beatty, Qiana L. Matthews and Yizhe Tang for useful advice and fruitful discussions. We are grateful to Cynthia M. Rodenburg for technical support of transmission electron microscopy at High Resolution Imaging Facility in University of Alabama at Birmingham. This work was supported by grants from the National Institutes of Health 5R01CA111569 (Dr. David T. Curiel), 5R56CA094084 (Drs. Masato Yamamoto and David T. Curiel) and by a grant from Susan G. Komen for the Cure PDF0707736 (Dr. Hideyo Ugai).

References

1. Haj-Ahmad Y, Graham FL. Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. *J Virol* 1986;57:267–274. [PubMed: 3001349]
2. Alemany R. Cancer selective adenoviruses. *Mol Aspects Med* 2007;28:42–58. [PubMed: 17300834]
3. Chiocca EA. Oncolytic viruses. *Nat Rev Cancer* 2002;2:938–950. [PubMed: 12459732]
4. Nettelbeck DM. Cellular genetic tools to control oncolytic adenoviruses for virotherapy of cancer. *J Mol Med* 2008;86:363–377. [PubMed: 18214411]
5. Russell SJ, Peng KW. Viruses as anticancer drugs. *Trends Pharmacol Sci* 2007;28:326–333. [PubMed: 17573126]
6. Ganly I, Kirn D, Eckhardt G, Rodriguez GI, Soutar DS, Otto R, Robertson AG, Park O, Gulley ML, Heise C, Von Hoff DD, Kaye SB. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 2000;6:798–806. [PubMed: 10741699]
7. Kirn D. Oncolytic virotherapy for cancer with the adenovirus dl1520 (Onyx-015): results of phase I and II trials. *Expert Opin Biol Ther* 2001;1:525–538. [PubMed: 11727523]
8. Ries SJ, Brandts CH. Oncolytic viruses for the treatment of cancer: current strategies and clinical trials. *Drug Discov Today* 2004;9:759–768. [PubMed: 15450242]
9. Liu TC, Kirn D. Gene therapy progress and prospects cancer: oncolytic viruses. *Gene Ther* 2008;15:877–884. [PubMed: 18418413]
10. Cherubini G, Petouchoff T, Grossi M, Piersanti S, Cundari E, Saggio I. E1B55K-deleted adenovirus (ONYX-015) overrides G1/S and G2/M checkpoints and causes mitotic catastrophe and endoreduplication in p53-proficient normal cells. *Cell Cycle* 2006;5:2244–2252. [PubMed: 16969092]
11. O'Shea CC, Johnson L, Bagus B, Choi S, Nicholas C, Shen A, Boyle L, Pandey K, Soria C, Kunich J, Shen Y, Habets G, Ginzinger D, McCormick F. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell* 2004;6:611–623. [PubMed: 15607965]
12. Sauthoff H, Pipiya T, Heitner S, Chen S, Bleck B, Reibman J, Chang W, Norman RG, Rom WN, Hay JG. Impact of E1a modifications on tumor-selective adenoviral replication and toxicity. *Mol Ther* 2004;10:749–757. [PubMed: 15451459]
13. Parato KA, Senger D, Forsyth PA, Bell JC. Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 2005;5:965–976. [PubMed: 16294217]
14. Le LP, Everts M, Dmitriev IP, Davydova JG, Yamamoto M, Curiel DT. Fluorescently labeled adenovirus with pIX-EGFP for vector detection. *Mol Imaging* 2004;3:105–116. [PubMed: 15296675]
15. Le LP, Le HN, Dmitriev IP, Davydova JG, Gavrikova T, Yamamoto S, Curiel DT, Yamamoto M. Dynamic monitoring of oncolytic adenovirus in vivo by genetic capsid labeling. *J Natl Cancer Inst* 2006;98:203–214. [PubMed: 16449680]
16. Le LP, Le HN, Nelson AR, Matthews DA, Yamamoto M, Curiel DT. Core labeling of adenovirus with EGFP. *Virology* 2006;351:291–302. [PubMed: 16678874]
17. Meulenbroek RA, Sargent KL, Lunde J, Jasmin BJ, Parks RJ. Use of adenovirus protein IX (pIX) to display large polypeptides on the virion--generation of fluorescent virus through the incorporation of pIX-GFP. *Mol Ther* 2004;9:617–624. [PubMed: 15093192]

18. Parks RJ. Adenovirus protein IX: a new look at an old protein. *Mol Ther* 2005;11:19–25. [PubMed: 15585402]
19. Vellinga J, Van der Heijdt S, Hoebe RC. The adenovirus capsid: major progress in minor proteins. *J Gen Virol* 2005;86:1581–1588. [PubMed: 15914835]
20. Crossland LD, Raskas HJ. Identification of adenovirus genes that require template replication for expression. *J Virol* 1983;46:737–748. [PubMed: 6854739]
21. Matsui T, Murayama M, Mita T. Adenovirus 2 peptide IX gene is expressed only on replicated DNA molecules. *Mol Cell Biol* 1986;6:4149–4154. [PubMed: 3025639]
22. Venkatesh LK, Chinnadurai G. Activation of the adenovirus 2 protein IX promoter by DNA replication in a transient expression assay. *Nucleic Acids Res* 1987;15:2235–2250. [PubMed: 3031591]
23. Vales LD, Darnell JE Jr. Promoter occlusion prevents transcription of adenovirus polypeptide IX mRNA until after DNA replication. *Genes Dev* 1989;3:49–59. [PubMed: 2523329]
24. Lee TW, Blair GE, Matthews DA. Adenovirus core protein VII contains distinct sequences that mediate targeting to the nucleus and nucleolus, and colocalization with human chromosomes. *J Gen Virol* 2003;84:3423–3428. [PubMed: 14645923]
25. Ugai H, Borovjagin AV, Le LP, Wang M, Curiel DT. Thermostability/infectivity defect caused by deletion of the core protein V gene in human adenovirus type 5 is rescued by thermo-selectable mutations in the core protein X precursor. *J Mol Biol* 2007;366:1142–1160. [PubMed: 17208253]
26. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol* 1996;70:4805–4810. [PubMed: 8676512]
27. Tang Y, Le LP, Matthews QL, Han T, Wu H, Curiel DT. Derivation of a triple mosaic adenovirus based on modification of the minor capsid protein IX. *Virology* 2008;377:391–400. [PubMed: 18570971]
28. Takahashi E, Cohen SL, Tsai PK, Sweeney JA. Quantitation of adenovirus type 5 empty capsids. *Anal Biochem* 2006;349:208–217. [PubMed: 16360111]
29. Greber UF, Willetts M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* 1993;75:477–486. [PubMed: 8221887]
30. Flint, SJ.; Enquist, LW.; Krug, RM.; Racaniello, VR.; Skalka, AM. Virus cultivation, detection, and genetics. In: Flint, SJ.; Enquist, LW.; Krug, RM.; Racaniello, VR.; Skalka, AM., editors. *Principles of virology: molecular biology, pathogenesis, and control*. ASM press; Washington: 2000. p. 25-56.
31. Baens M, Noels H, Broeckx V, Hagens S, Fevery S, Billiau AD, Vankelecom H, Marynen P. The dark side of EGFP: defective polyubiquitination. *PLoS ONE* 2006;1:e54. [PubMed: 17183684]
32. Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang CC, Kain SR. Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 1998;273:34970–34975. [PubMed: 9857028]
33. Kornuc M, Kliever S, Garcia J, Harrich D, Li C, Gaynor R. Adenovirus early region 3 promoter regulation by E1A/E1B is independent of alterations in DNA binding and gene activation of CREB/ATF and AP1. *J Virol* 1990;64:2004–2013. [PubMed: 2139139]
34. Hurst HC, Jones NC. Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. *Genes Dev* 1987;1:1132–1146. [PubMed: 2828166]
35. Weeks DL, Jones NC. Adenovirus E3-early promoter: sequences required for activation by E1A. *Nucleic Acids Res* 1985;13:5389–5402. [PubMed: 2991862]
36. Holm PS, Lage H, Bergmann S, Jurchott K, Glockzin G, Bernshausen A, Mantwill K, Ladhoff A, Wichert A, Mymryk JS, Ritter T, Dietel M, Gansbacher B, Royer HD. Multidrug-resistant cancer cells facilitate E1-independent adenoviral replication: impact for cancer gene therapy. *Cancer Res* 2004;64:322–328. [PubMed: 14729641]
37. Steinwaerder DS, Carlson CA, Lieber A. DNA replication of first-generation adenovirus vectors in tumor cells. *Hum Gene Ther* 2000;11:1933–1948. [PubMed: 10986565]
38. Nelson JE, Kay MA. Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J Virol* 1997;71:8902–8907. [PubMed: 9343256]
39. Matthews DA. Adenovirus protein V induces redistribution of nucleolin and B23 from nucleolus to cytoplasm. *J Virol* 2001;75:1031–1038. [PubMed: 11134316]

40. Fukuda K, Abei M, Ugai H, Seo E, Wakayama M, Murata T, Todoroki T, Tanaka N, Hamada H, Yokoyama KK. E1A, E1B double-restricted adenovirus for oncolytic gene therapy of gallbladder cancer. *Cancer Res* 2003;63:4434–4440. [PubMed: 12907616]
41. Zhai ZH, Nickerson JA, Krochmalnic G, Penman S. Alterations in nuclear matrix structure after adenovirus infection. *J Virol* 1987;61:1007–1018. [PubMed: 3820360]
42. Dunker R, Brown DT. Tubular subviral structure produced in adenovirus-infected KB cells. *J Virol* 1979;31:568–574. [PubMed: 480478]
43. Ornelles DA, Shenk T. Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. *J Virol* 1991;65:424–429. [PubMed: 1824641]
44. Ugai H, Yamasaki T, Hirose M, Inabe K, Kujime Y, Terashima M, Liu B, Tang H, Zhao M, Murata T, Kimura M, Pan J, Obata Y, Hamada H, Yokoyama KK. Purification of infectious adenovirus in two hours by ultracentrifugation and tangential flow filtration. *Biochem Biophys Res Commun* 2005;331:1053–1060. [PubMed: 15882984]
45. Ternovoi VV, Le LP, Belousova N, Smith BF, Siegal GP, Curiel DT. Productive replication of human adenovirus type 5 in canine cells. *J Virol* 2005;79:1308–1311. [PubMed: 15613357]
46. Le LP, Li J, Ternovoi VV, Siegal GP, Curiel DT. Fluorescently tagged canine adenovirus via modification with protein IX-enhanced green fluorescent protein. *J Gen Virol* 2005;86:3201–3208. [PubMed: 16298964]
47. Condit, RC. Principles of Virology. In: Knipe, DM.; Howley, PM., editors. *Fundamental Virology*. Vol. 4. Lippincott Williams & Wilkins; Philadelphia: 2001. p. 19-51.
48. Maizel JV Jr, White DO, Scharff MD. The polypeptides of adenovirus. I Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 1968;36:115–125. [PubMed: 5669982]
49. Saban SD, Silvestry M, Nemerow GR, Stewart PL. Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J Virol* 2006;80:12049–12059. [PubMed: 17005667]
50. Fabry CM, Rosa-Calatrava M, Moriscot C, Ruigrok RW, Boulanger P, Schoehn G. The C-terminal domains of adenovirus serotype 5 protein IX assemble into an antiparallel structure on the facets of the capsid. *J Virol* 2009;83:1135–1139. [PubMed: 19004948]
51. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods* 2005;2:905–909. [PubMed: 16299475]
52. Vayda ME, Rogers AE, Flint SJ. The structure of nucleoprotein cores released from adenovirions. *Nucleic Acids Res* 1983;11:441–460. [PubMed: 6828374]
53. Matthews DA, Russell WC. Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J Gen Virol* 1998;79 (Pt 7):1671–1675. [PubMed: 9680130]
54. Chatterjee PK, Vayda ME, Flint SJ. Interactions among the three adenovirus core proteins. *J Virol* 1985;55:379–386. [PubMed: 4020954]
55. van Oostrum J, Burnett RM. Molecular composition of the adenovirus type 2 virion. *J Virol* 1985;56:439–448. [PubMed: 4057357]
56. Lehmborg E, Traina JA, Chakel JA, Chang RJ, Parkman M, McCaman MT, Murakami PK, Lahidji V, Nelson JW, Hancock WS, Nestaas E, Pungor E Jr. Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *J Chromatogr B Biomed Sci Appl* 1999;732:411–423. [PubMed: 10517364]
57. Chatterjee PK, Vayda ME, Flint SJ. Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled in vivo. *J Mol Biol* 1986;188:23–37. [PubMed: 3712442]
58. Anderson CW, Young ME, Flint SJ. Characterization of the adenovirus 2 virion protein, mu. *Virology* 1989;172:506–512. [PubMed: 2800334]
59. Meier O, Boucke K, Hammer SV, Keller S, Stidwill RP, Hemmi S, Greber UF. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol* 2002;158:1119–1131. [PubMed: 12221069]

60. Cascallo M, Capella G, Mazo A, Alemany R. Ras-dependent oncolysis with an adenovirus VAI mutant. *Cancer Res* 2003;63:5544–5550. [PubMed: 14500393]
61. Thimmappaya B, Weinberger C, Schneider RJ, Shenk T. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. *Cell* 1982;31:543–551. [PubMed: 6297772]
62. Chee-Sheung CC, Ginsberg HS. Characterization of a temperature-sensitive fiber mutant of type 5 adenovirus and effect of the mutation on virion assembly. *J Virol* 1982;42:932–950. [PubMed: 7097864]
63. Bhat BM, Brady HA, Wold WS. Virus deletion mutants that affect a 3' splice site in the E3 transcription unit of adenovirus 2. *Mol Cell Biol* 1985;5:2405–2413. [PubMed: 3879768]
64. Mahr JA, Boss JM, Gooding LR. The adenovirus e3 promoter is sensitive to activation signals in human T cells. *J Virol* 2003;77:1112–1119. [PubMed: 12502827]
65. Garcia J, Wu F, Gaynor R. Upstream regulatory regions required to stabilize binding to the TATA sequence in an adenovirus early promoter. *Nucleic Acids Res* 1987;15:8367–8385. [PubMed: 2959908]
66. Bett AJ, Prevec L, Graham FL. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 1993;67:5911–5921. [PubMed: 8371349]
67. Knipe, David M.; Samuel, Carles E.; Palese, P. Virus-Host cell interaction. In: Knipe, DM.; Howley, PM., editors. *Fundamental Virology*. Vol. 4. Lippincott Williams & Wilkins; Philadelphia: 2001.
68. Webster A, Russell S, Talbot P, Russell WC, Kemp GD. Characterization of the adenovirus proteinase: substrate specificity. *J Gen Virol* 1989;70 (Pt 12):3225–3234. [PubMed: 2691633]
69. Ruzindana-Umunyana A, Imbeault L, Weber JM. Substrate specificity of adenovirus protease. *Virus Res* 2002;89:41–52. [PubMed: 12367749]
70. Stewart, PL. Adenovirus structure. In: Curiel, DT.; Douglas, JT., editors. *Adenoviral vectors for gene therapy*. Academic Press; London, UK: 2002.
71. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoebe RC, Van Der Eb AJ. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* 1996;7:215–222. [PubMed: 8788172]
72. Belousova N, Korokhov N, Krendelshchikova V, Simonenko V, Mikheeva G, Triozzi PL, Aldrich WA, Banerjee PT, Gillies SD, Curiel DT, Krasnykh V. Genetically targeted adenovirus vector directed to CD40-expressing cells. *J Virol* 2003;77:11367–11377. [PubMed: 14557622]
73. Kanegae Y, Makimura M, Saito I. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol* 1994;47:157–166. [PubMed: 7823411]
74. Saito I, Oya Y, Yamamoto K, Yuasa T, Shimojo H. Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. *J Virol* 1985;54:711–719. [PubMed: 3999192]

Fig. 1.

Schematic representation of Ad5 and fluorescently labeled adenoviruses used in this study. IX; the gene for protein IX, V; the gene for protein V. Ad5 is human adenovirus type 5 generated using pTG3602. Ad5-wt-IX-EGFP and Ad5-wt-IX-mRFP1 are capsid labeled adenovirus mutants with pIX-EGFP and pIX-mRFP1, respectively. The genes for pIX-EGFP and pIX-mRFP1 are inserted in each genome instead of the native IX gene. EGFP; the gene for an enhanced green fluorescent protein, mRFP1; the gene for a monomeric red fluorescent protein 1. Ad5-dE1-IX-mRFP1 is an E1-deleted adenovirus mutant with pIX-mRFP1 and the native IX gene is replaced to the pIX-mRFP1 gene. Ad5-E3-V-EGFP is an adenovirus mutant with the V-EGFP gene in the deleted E3 region. Ad5-IX-mRFP1-E3-V-EGFP is a fluorescently labeled adenovirus mutant with pIX-mRFP1 and pV-EGFP generated in this study.

Fig. 2.

Expression of fluorescent proteins in infected cells with the fluorescently labeled adenoviruses formed an individual plaque. Plaque assay was performed as described in the Materials and Methods section. Monolayers of A549 cells in 6-well plate were infected with adenoviruses, overlaid with 0.75% agar in growth medium containing 2% FBS, and stained with 0.33% neutral red at 14 d.p.i. Microscopic view (magnification, $\times 200$) of individual plaques formed on A549 cells which were infected by Ad5 and the fluorescently labeled adenoviruses, and the signals of fluorescent proteins were detected on a plaque by fluorescent microscopy.

Fig. 3.

Analysis of protein composition of fluorescently labeled viral particles by gel-staining and western blot. (a) Detection of adenoviral proteins incorporated in the particles. 3×10^{10} VP equivalents of purified adenoviruses were run on a 10% (w/v) SDS-PAGE and the gel was stained with GELCODE Blue reagent. Protein molecular mass makers (in kilodaltons) are indicated on the left. (b) Western blot analysis of pIX from purified adenoviral particles. The structural proteins (3×10^{10} VP equivalents) of purified adenoviruses were separated by 12% SDS-PAGE and western blot analysis was performed with anti-pIX antibody on a nitrocellulose membrane. Molecular mass markers were indicated kilodaltons on the left side. Arrows show non-specific bands and asterisks show proteolytic products of fluorescently labeled pIX. (c)

1.0×10^{10} VP of purified adenoviruses were separated by 10% SDS-PAGE and blotted with anti-pV antibody. Molecular mass markers were indicated kilodaltons on the left side.

Fig. 4. Negatively stained electron micrographs of purified Ad5 (a), Ad5-wt-IX-EGFP (b), Ad5-wt-IX-mRFP1(c), Ad5-E3-V-EGFP and Ad5-IX-mRFP1-E3-V-EGFP (e). Black arrows show the viral particles incorporated aggregated proteins. Scale bar shows 100 nm. Magnification: (a and b) 52,000 $\times$, (c) 67,000 $\times$ and (d and e) 100,000 $\times$.

Fig. 5.

In vitro visualization of fluorescently labeled adenoviruses. Viral solution included 1.0×10^9 VP was spotted on a glass slide and sealed with a cover glass and the fluorescent signals of adenoviral particles contained fluorescently labeled proteins were detected by fluorescent microscope. (a) and (b) Negative control, Ad5, (c) Ad5-wt-IX-EGFP, (d) Ad5-wt-IX-mRFP1, (e) Ad5-E3-V-EGFP, (f-h) Ad5-IX-mRFP1-E3-VEGFP. Arrows show adenoviral particles containing both of pIX-mRFP1 and pV-EGFP. Panels (a), (c), (e) and (g) were detected by green filter and panels (b), (d) and (f) were detected by red filter. Panel (h) was merged with panels (f) and (g) by Adobe Photoshop CS2.

Figure 6a

(b)

Figure 6b**Fig. 6.**

Tracking of fluorescently labeled adenoviral particles. Fluorescently labeled adenovirus infected A549 cells at MOIs of 10,000 VP/cell (a) and 50,000 VP/cell (b) with growth medium containing 2% FBS. After incubation at 37 °C for 10 min, the infected cells were fixed and stained with Hoechst 33342, and the signals for fluorescently labeled adenoviral particles were visualized by fluorescent microscopy (magnification, $\times 1000$). (a) White arrow and orange arrows show pV-EGFP and pIX-mRFP1 detected in the nucleoplasm, respectively. (b) Arrows show the detection of pIX-mRFP1 and pV-EGFP at the adjacent positions in infected cells after 10 m.p.i. Hoechst 33342 stain showed blue color.

Fig. 7.

Characterization of fluorescently labeled adenoviruses. (a) One-step growth curve analyses were performed with Ad5 and four kinds of fluorescently labeled adenoviruses in A549 cells at an MOI of 10 PFU/cell. Values are means $\pm$ SD of results from triplicate experiments. (b) Quantification of the cellular uptake of the adenoviral genome in infected cells. Adenovirus infected A549 cells at an MOI 10 PFU/cell and total DNA extracted from infected cells at 1 h.p.i. was quantified using the E4 primers to determine the copy number of the adenoviral genome. Values are means $\pm$ SD of results from triplicate experiments. (c) The replication of the adenoviral genome in A549 cells infected by adenovirus at an MOI 10 of PFU/cell followed by cell harvesting at various time points post-infection. Total DNA was extracted from infected cells and the copy number of the adenoviral genome was quantified by qPCR using the E4 primers and the total DNA as a template. Values are means $\pm$ SD of results from triplicate experiments. (d and e) Western blot analysis of the expression profiles of pIX (d) and pV (e) labeled by fluorescent proteins. Cells infected by adenovirus were harvested at various times post-infection and the crude protein was prepared as described in the Materials and Methods section. 10 μ g of the crude protein was separated by SDS-PAGE, and pIX and pV which were expressed in infected cells were detected using the indicated antibodies. SDS-PAGE and western blot analysis were performed under the same conditions.

(a)

Figure 8a

Figure 8b

Figure 8c

Fig. 8.

In vitro detection of fluorescently labeled proteins in infected cells (a) The expression profiles of pIX-mRFP1 in cells infected with a replication-deficient or competent adenovirus containing a fluorescently labeled pIX. A549 cells were infected by Ad5-dE1-IX-mRFP1 or Ad5-wt-IX-mRFP1 at an MOI 10 PFU/cell and the fluorescent signal for pIX-mRFP1 was detected at various times post-infection by fluorescent microscopy with the Olympus DP1 digital camera. (b) Detection of pIX-mRFP1 and pV-EGFP in cells infected with Ad5-IX-mRFP1-E3-V-EGFP by fluorescent microscopy. A549 cells were infected by Ad5 or Ad5-IX-mRFP1-E3-V-EGFP at an MOI 10 PFU/cell and the fluorescent signals for pIX-mRFP1 and pV-EGFP were detected at various times post-infection by fluorescent microscopy (magnification, $\times 200$). Arrows show

cells expressing pV-EGFP and pIX-mRFP1 observed at 12 and 24 h.p.i., respectively. (c) Detection of pIX-EGFP, pIX-mRFP1 and pV-EGFP in cells infected with Ad5-wt-IX-EGFP, Ad5-wt-IX-mRFP1 and Ad5-E3-V-EGFP by fluorescent microcopy. A549 cells were infected by the fluorescently labeled adenovirus at an MOI of 10 PFU/cell and the fluorescent signals for pIX-EGFP, pIX-mRFP1 and pV-EGFP were detected at various times post-infection by fluorescent microcopy (magnification, $\times 200$). Arrows shows cells expressing pV-EGFP observed at 12 h.p.i.

Fig. 9.

Subcellular localization of fluorescently labeled pIX and pV. A549 cells were infected by adenoviruses at an MOI of 10 PFU/cell for 18 h.p.i. After the infected cells were fixed with PBS contained 10% formalin, Hoechst 33342 staining was performed for 10 min before mounting and sealing. The fluorescently labeled proteins of pIX and pV were detected by fluorescent microscopy (magnification, $\times 1000$). (a)-(c) Ad5-wt-IX-EGFP, (d)-(f) Ad5-wt-IX-mRFP1, (g)-(i) Ad5-E3-V-EGFP, (j)-(m) Ad5-IX-mRFP1-E3-V-EGFP. Arrows and arrowheads show the aggregation of pIX-EGFP observed in the cytoplasm of infected cells and the localization of pV-EGFP detected in the nucleoplasm, respectively.

Figure 10a

Figure 10b

Figure 10c

Fig. 10.

Translocation of pIX and pV at the late stage of infection. (a) Ad5-IX-mRFP1-E3-V-EGFP infected A549 cells at an MOI of 10 PFU/cell, and the fluorescently labeled proteins expressed in infected cells were detected at various times post-infection by fluorescent microscopy (magnification, $\times 1000$). After fixation of the infected cells with PBS contained 10% formalin, DNA staining was performed using Hoechst 33342 for 10 min before mounting and sealing. (i)-(iv) Boxes display enlarged areas. White arrows show the fluorescent flecks for pV-EGFP observed in the cytoplasm. Orange arrows show the fluorescent signals for pV-EGFP observed in the nuclear membrane. (b) Translocation of pIX during the late stage of infection. Ad5-wt-IX-EGFP or Ad5-wt-IX-mRFP1 infected A549 cells at an MOI of 10 PFU/cell. pIX-EGFP

and pIX-mRFP1 expressed in infected cells were detected at various times post-infection by fluorescent microscopy (magnification, $\times 1000$). Arrows show the fluorescent speckles for pIX-EGFP and pIX-mRFP1 observed in the nucleoplasm. (c) Translocation of pV during the late stage of infection. Ad5-E3-V-EGFP was infected at an MOI of 10 PFU/cell in A549 cells, and pV-EGFP expressed in infected cells was detected at various times post-infection by fluorescent microscopy (magnification, $\times 1000$). Arrows show the fluorescent speckles for pV-EGFP observed in the cytoplasm.

Table 1

Titration of Ad5 and fluorescently labeled adenoviruses

Virus	Genome size (bp) ^{a,b}	VP/ml	PFU/ml	FCU ^c /ml	VP/VPFU	FCU/VPFU	VP/FCU
Ad5	35,938	4.94 × 10 ¹¹	1.58 × 10 ¹⁰ ± 7.68 × 10 ⁸	-	31	-	-
Ad5-wt-IX-EGFP	36,984	1.11 × 10 ¹²	6.67 × 10 ⁸ ± 2.89 × 10 ⁸	1.08 × 10 ¹⁰ ± 1.61 × 10 ⁹	1,662	16	103
Ad5-wt-IX-mRFP1	36,653	2.09 × 10 ¹²	2.17 × 10 ⁹ ± 1.53 × 10 ⁹	2.13 × 10 ¹⁰ ± 2.02 × 10 ⁹	966	10	98
Ad5-dE1-IX-mRFP1	32,887	3.67 × 10 ¹¹	1.50 × 10 ⁸ ± 1.53 × 10 ⁷	4.33 × 10 ⁸ ± 2.89 × 10 ⁷	2,447	3	846
Ad5-E3-V-EGFP	35,497	5.71 × 10 ¹¹	1.03 × 10 ¹⁰ ± 3.18 × 10 ⁹	2.22 × 10 ¹⁰ ± 5.39 × 10 ⁹	55	2	26
Ad5-IX-mRFP1-E3-V-EGFP	36,158	4.38 × 10 ¹¹	5.17 × 10 ⁹ ± 1.04 × 10 ⁹	4.33 × 10 ⁹ ± 2.89 × 10 ⁸	85	1	101

^abp, base pair

^bThe genome sizes of adenoviruses were calculated based on the nucleotide sequences of the wild type Ad5 genome (Genbank Accession No. AC_000008), the EGFP gene (Genbank Accession No. U55762), the mRFP1 gene (Genbank Accession No. AF506027).

^cFCU; fluorescent cell unit

^d911 cells were used to determine FCU and PFU titers.