Quantitative Microscopy of Fluorescent Adenovirus Entry

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Fluorescence imaging of cells is a powerful tool for exploring the dynamics of organelles, proteins, and viruses. Fluorescent adenoviruses are a model system for cargo transport from the cell surface to the nucleus. Here, we describe a procedure to quantitate adenovirus-associated fluorescence in different subcellular regions. CCD camera-captured fluorescence sections across entire cells were deblurred by a fast Fourier transformation, the background was subtracted images merged, and virus fluorescence quantitated. The validity of the deblurring routine was verified by confocal laser scanning microscopy, demonstrating that objects were neither generated nor deleted. Instead, the homogeneity of both the average intensity and the size of fluorescent particles was increased, facilitating automated quantification. We found that nuclear fluorescence of wt adenovirus, but not of a virus mutant ts1, which fails to escape from endosomes, was maximal at 90 min postinfection (p.i.). Surprisingly, nuclear fluorescence decreased at 120 min, but increased again at 240 min p.i., suggesting that wt virus targeting to the nucleus may be multiphasic and regulated. Interestingly, only the first nuclear transport period of wt but not ts1 virus coincided with a significant increase of the peripheral and decrease of the cytoplasmic regions, indicative of signal-dependent cell contraction.

Key Words: adenovirus transport; cytoplasm; deblurring; fluorescent virus; image processing; nucleus; plasma membrane; quantitative microscopy; thresholding.

INTRODUCTION

Fluorescence imaging of live and chemically fixed cells is an increasingly powerful tool for the subcellular analysis of cell and organelle dynamics, signaling, and gene expression (De Giorgi et al., 1999; Ludin and Matus, 1998; Raz et al., 1998; Tvarusk et al., 1999). Tagging the green fluorescent protein (GFP)2 and color variants thereof to a growing number of proteins combined with sensitive noninvasive imaging technologies has further broadened the scope of fluorescence cell analysis (Tsien, 1998). Examples of protein dynamics in time and space include the nucleo-cytoplasmic trafficking of the calcium sensor calmodulin (Crask et al., 1999; Li et al., 1999), the transient membrane localization of protein kinase B during activation of the B cell receptor in lymphocytes (Astoul et al., 1999) or of protein kinase C following local changes in lipid composition of cell membranes (Oancea et al., 1998). In addition, static analysis of GFP-expressing non-fixed cells in the developing wing of drosophila has recently led to the discovery of a new actin-based structure, which is implicated in long-range communication between cells (Ramirez-Weber and Kornberg, 1999).

Fluorescent viruses have long been used as probes of cell function (for review, see Stidwill and Greber, 2000). Initially, rhodamine-labeled adenovirus type 2 (Ad) and reovirus particles were used to study vector attachment to cultured cells (Defer et al., 1990; Georgi et al., 1990; Persson et al., 1983). Subsequent virus entry and cell infection experiments have indicated that fluorophore-tagged Ad particles can be prepared without affecting their infectivity (Greber et al., 1998; Greber et al., 1997). Ads are nonenveloped, icosahedral particles of about 90 nm diameter (Burnett, 1997). The main capsid component is the facetted-associated hexon protein. Hexon is assembled and stabilized by various minor proteins and largely protects the double-stranded linear DNA genome, which is packed inside the capsid together with additional viral proteins, including the cysteine protease L3/p23. Hexon remains

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2 Abbreviations used: Ad, adenovirus type 2; CCD, charge-coupled device; CLSM, confocal laser scanning microscopy; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; FFT, fast Fourier transformation; FITC, fluorescein isothiocyanate; FM, fluorescence microscopy; GFP, green fluorescent protein; MT, microtubule; p.i., postinfection; PM, plasma membrane; r.o.i., region(s) of interest; SEM, standard error of mean; TR, Texas red; ts, temperature sensitive; wt, wild type.
associated with the viral DNA until the particles reach the nucleus of a target cell and dissociate (Greber et al., 1996). The base of the capsid vertexes is built of the penton and the protruding fibers and primarily functions during virus entry (Greber, 1998). Entry occurs by a dual receptor mechanism involving high affinity attachment of the terminal knob domain of the fiber protein to the immunoglobulin-related cell surface receptor coxsackie–Ad receptor (see Bergelson, 1999), followed by contacts of penton base with fibronectin/vitronectin binding integrins (Wickham et al., 1993). An acid-assisted endosome escape mechanism releases virus particles into the cytosol (Greber, 1998). In addition, virus escape from endosomes requires a fully processed capsid structure lacking in ts1 (Miles et al., 1980; Weber, 1976). Ts1 has a point mutation in the L3/p23 protease gene preventing protease packaging and processing of the viral capsid structure (for review, see Greber, 1998). Incoming ts1 virions are confined to endosomes and eventually degraded in lysosomes. Like cytosolic wt virus, ts1 is transported to and from the perinuclear region by microtubule-dependent motors, as reported in live cell experiments using fluorescent viruses (Suomalainen et al., 1999). wt Ad but not ts1 then associates with the nuclear envelope, where it specifically attaches to nuclear pore complexes and releases its DNA into the nucleoplasm (Greber et al., 1996, 1997).

Fluorescent virus particles are not only useful for analyzing the cell biology of virus entry, but also for facilitating analysis of vector tropism, which is often determined by the availability and localization of receptors on the cell surface (Walters et al., 1999). To effectively harness the potential of fluorescent viruses, quantitative procedures are indispensable. Here, we describe a protocol to quantitate Ad-associated fluorescence in different regions of fixed cells at various times postinfection (p.i.). Stacks of fluorescent microscopy images of infected cells were captured with a CCD camera, deblurred, and merged and the fluorescence was quantitated in r.o.i., which were generated by thresholding of a fluorescence marker image. Our results show that both wt Ad and mutant ts1 progressively enriched in the perinuclear region, albeit with different kinetics. While the endosomal ts1 virus reached quasi-steady state levels near the nucleus at 30 min p.i. cytosolic wt virus was transported to the nucleus in two subsequent waves. The first wave lasted until 90 min p.i. and was followed by a period of virus transport to the periphery, followed by the second nuclear transport period from 120 to 240 min p.i. Surprisingly, the first, but not the second nuclear transport period of wt Ad coincided with significant cell contraction, which was reversed at 120 min p.i. These results raise the possibility that nuclear transport of wt Ad may be regulated by virus-induced cell signaling. We expect that the protocol described here is applicable to any other fluorescent object of regular shape, including naked and lipid-enveloped viruses and cell organelles.

MATERIALS AND METHODS

Cells and viruses. HeLa, A549, and TC7 cells were grown on 12-mm glass coverslips (0.13–0.16 mm thickness, Assistant, Germany) in DMEM–F12 medium in the presence of 7% fetal bovine serum and 5% CO2 and infected with purified Ad as described (Suomalainen et al., 1999). Labeling of isolated Ad with Texas red (TR) and quantification of fluorophore incorporation into SDS-dissociated Ad proteins by SDS–PAGE and phosphoimaging were performed as described (Greber et al., 1998).

Microinjections. Purified Ad–TR (0.8 mg/ml) was diluted 20-fold into 0.01 M Tris–HCl, pH 7.4, 0.12 M KCl containing 1 mg/ml aldehyde-fixable 10-kDa Dextran–FITC (Molecular Probes, Leiden NE), centrifuged at 10 000 g for 5 min, and microinjected into the cytoplasm of HeLa cells in RPMI medium containing 20 mM HEPES–NaOH, pH 7.4, and 0.2% BSA, using the Eppendorf microinjection system at constant ejection pressure (injector 5242, manipulator 5120). Microinjection needles were pulled by a Narishige needle puller (model PB-7, Nikon, Switzerland). To reduce virus uptake by the natural pathway, HeLa cells were pretreated with 0.4 µg/ml of recombinant Ad fiber knob in DMEM–F12 medium containing 0.2% BSA for 30 min at 37°C. This procedure effectively decreased virus uptake by more than 90%, as indicated by binding studies with radioactively labeled Ad (data not shown).

Virus uptake and cell staining. Briefly, 0.16 µg of purified Ad–TR (approximately 1.4 × 107 particles) were attached to the surface of 7 × 106 cells in cold RPMI–BSA medium for 60 min as described (Suomalainen et al., 1999). Unbound virus (about 90 to 95% of the input virus) was washed off and cells were incubated in DMEM–BSA medium at 37°C for indicated amounts of time. Cells were quickly washed in PBS at 37°C and fixed immediately in 3.3% pFA in PBS for 12 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 in PBS for 2 min to reduce autofluorescence, quenched in PBS containing 25 mM NH4Cl, and stained with an anti-plasma membrane Ca2+ ATPase 1 antiseraum (Stauffer et al., 1995) and goat anti-rabbit IgG conjugated to Alexa488 (Molecular Probes). The nucleus was stained with DAPI (0.5 µg/ml, Sigma, Switzerland) for 5 min and the sample was mounted onto a glass slide in 85% glycerol containing 20 mM Tris–HCl, pH 8.8, and 2 mg/ml para-phenylenediamine.

Analysis of isolated virus by fluorescence microscopy (FM). Isolated Ad–TR was diluted to 8 µg/ml into Hanks’ medium containing 2 mg/ml BSA to yield a virus particle density of about 7.2 × 1010 per µl (see Greber et al., 1998). Twelve microliters of virus solution was placed on a microscope slide and furnished with a coverslip (18 × 18 mm), such that the spacing between the glass surfaces was approximately 35 µm. The coverslip was sealed with nail polish and virus particles were analyzed immediately by fluorescence microscopy using a 100× oil immersion objective (Leica, Switzerland) and the CCD camera specified below. Images were recorded in five subsequent planes at 0.5-µm intervals starting from the bottom of the coverslip. Bottom and top images were discarded and the remaining three images either merged directly to a new raw image or were individually processed by fast Fourier transformation (FFT) and then merged using the sum function of the MetaMorph software (see below). Fluorescent objects were further selected by a thresholding function and analyzed in regions of 200 × 250 pixels (30 × 37.5 µm) by quantitation of pixel intensities and object sizes.
**RESULTS**

**Fluorescence Labeling of Isolated Ad and Analysis by Fluorescence Microscopy**

We have prepared Texas red-labeled wt and ts1 mutant Ad following a protocol described for fluorescein and TR labeling of wt Ad (Greber et al., 1998). Fluorography and Coomassie blue staining of SDS–PAGE-fractionated virus particles indicated that more than 90% of the TR fluorescence was associated with hexon in both wt and ts1 particles (Fig. 1). Only a minor fraction of TR was associated with other virion proteins. This result was markedly different from fluorescein-modified wt Ad, where 50% of the fluorescein was incorporated into external capsid proteins other than hexon (Greber et al., 1998). Wt Ad contained on average 2000 TR molecules (about twice as much as TR-labeled ts1) and was fully infectious, as determined by plaque assays on A549 cells (not shown). Earlier studies have shown that hexon of both wt and ts1 particles remains associated with the viral genome until the particles have arrived at the cytosolic side of nuclear pore complexes (Greber, 1998; Suomalainen et al., 1999). We thus conclude that these TR-labeled virus preparations can be used to trace subcellular virus particles.

Isolated wt Ad particles were analyzed by fluorescence microscopy. Ad–TR (8 µg/ml, 7.2 × 10^6/µl) was placed between two glass surfaces for analysis by fluorescence microscopy with a cooled CCD camera (15 × 15-µm pixel size) as described under Materials and Methods. Three successive z-stacked images of 200 × 250 pixels were captured through a 100× objective lens at 0.5-µm intervals near the bottom of the sample and merged together to a combined raw image (Fig. 2A). As shown in the overview picture (inset), the image contained about 28 discrete objects, each consisting of 10 to 20 pixels. The image also contained a significant amount of blurred signals, probably due to out-of-focus information and nonuniform illumination. Based on size, intensity, and frequency, the fluorescent objects resembled authentic virus particles, which had been analyzed in live virus entry experiments (Suomalainen et al., 1999). We therefore subjected the individual images of the z-stack to fast Fourier transformation. FFT had previously proven useful in removing nonhomogeneous background, which was due to nonuniform illumination, autofluorescence, or out-of-focus information (Russ, 1995). The parameters were chosen such that none of the objects were removed and no object was added. The FFT-processed images were then merged. The resulting image had significantly less background information (Fig. 2B) and the pixels of its objects were homogeneous, as indicated by the average pixel intensity of the FFT-processed image.
To select for fluorescent objects and quantitate their gray levels and dimensions, we applied a thresholding function under conditions which neither added nor deleted any of these objects. The processed objects (indicated as a binary image in Fig. 2C) were predominantly homogeneous in terms of both gray values and sizes, as indicated by a frequency analysis of 13 different images containing 581 objects (Fig. 2D). More than 85% of the objects had gray levels less than 40% of the maximal object value, which was 7736, and more than 80% of the objects had sizes between 30 and 60% of the maximal object size, which was 24 pixels. The average object had a gray level of 2267 and a size of 11.35 pixels, respectively. The latter is equivalent to 0.25 µm², which is about 16 times larger than a mature virus particle observed by electron microscopy. We concluded that the fluorescent objects were single virus particles, since they were of a quasi-regular appearance with the highest fluorescence in the center and decreasing gray values toward the periphery in both the raw and the processed images (Figs. 2A and 2B). Furthermore, these objects were normally distributed in

FIG. 1. SDS–PAGE analysis of TR-labeled wt and ts1 Ad. Approximately 10 µg of trichloroacetic acid-precipitated TR-labeled wt and ts1 Ad was separated in a 4–12% acrylamide gradient SDS gel and analyzed by fluorography (lanes 1 and 2) and Coomassie blue staining, respectively (lanes 4 and 5), as described (Greber et al., 1998). About 20 µg of nonlabeled, isolated Ad was loaded as a control (lanes 3 and 6). Virion proteins are indicated on the right and relative molecular weights of marker proteins on the left (Gibco-BRL, BENCHMARK protein ladder).
terms of size and gray levels (Fig. 2D). The average object number per image was 44.7. This was within a twofold range of the theoretical number of virus particles per image, which was calculated to be 24.3, assuming that the merged optical sections were about 3 µm thick and the virus was homogeneously distributed between the glass plates. Possibly, virus particles were somewhat enriched at the surface of the glass coverslip where the sampling occurred. Together, this population analysis shows that the FFT and the thresholding procedures do not delete objects but efficiently remove background signals and thus generate a homogenous population of fluorescent virus particles.

**FM and CLSM Analysis of Microinjected Ad–TR**

To test if the applied FFT and thresholding operations were valid for an analysis of fluorescent virus particles within target cells, we microinjected isolated TR-labeled Ad into the cytosol of HeLa cells and analyzed the TR signal by fluorescence microscopy in the fixed cells 5 min postinjection. The same cells were also imaged by CLSM in order to assess the reliability of the imaging and processing procedures (Fig. 3). Prior to microinjection, cells were treated with recombinant fiber knob (Louis et al., 1994), which blocks the natural infectious pathway of Ad (Bergelson, 1999). To facilitate the identification of injected cells, we coinjected fluorescein-labeled 10-kDa Dextran together with Ad–TR (Fig. 3A, e). Two of the injected cells are indicated by arrows and one noninjected cell is pointed out by an arrowhead in the DIC image (Fig. 3A, d). TR fluorescence was recorded across the entire z-dimension as defined by dextran–FITC fluorescence using step intervals of 0.5 µm for FM and 0.1 µm for CLSM, respectively. Since the actual diameter of the virus capsid is approximately 100 nm (Stewart et al., 1993), the CLSM procedure most likely captured all the fluorescent virus particles present in the cell. The images of an entire z-stack were merged and displayed directly (Fig. 3A, a and f) or background-subtracted (Fig. 3A, b) and further processed by FFT (Fig. 3A, c). The distribution and number of virus particles were very similar in all cases, as seen when the thresholded fluorescent objects in one of the injected cells are counted (large arrow, Fig. 3A, d). In the raw and FFT-processed CLSM images, we found 55 and 58 virus particles, respectively, whereas the FFT-processed FM image contained 53 discrete objects (Fig. 3A, a, c, and f). The difference in the number of objects was most likely due to particles that were close together and, thus, not resolved by either the microscopy or the image-processing procedures. No discrete objects were, however, generated or deleted, and both the pixel values and the sizes of fluorescent particles had a normal distribution under all the conditions (Figs. 3B and 3C). Similar to the processing of extracellular virus particles (Fig. 2), the FFT procedure yielded a homogenous population of intracellular virus particles with respect to intensities and sizes. In the FM-generated images, more than 80% of the particles had gray levels and sizes

FIG. 2. Population analysis of isolated wt Ad-TR particles by FM and image processing. Isolated Ad–TR was placed between a microscope slide and a glass coverslip and imaged by FM in three subsequent planes of focus at 0.5-µm intervals. Images were merged using the summation function yielding the raw image (A). Raw images were FFT-processed (B) or FFT-processed and further thresholded giving a binarized image (C), which was used to select objects (virus particles). Bar equals 0.75 µm. (D) The number of fluorescent objects in the FFT-processed images plotted as a function of pixel intensities and apparent object size (inset). The maximal value of all the pixels in an object was 7736 and the maximal object size was 24 pixels.
FIG. 3. Verification of the FFT and thresholding procedures by CLSM with microinjected Ad–TR. HeLa cells were microinjected on glass coverslips into the cytoplasm with Ad–TR (40 ng/ml) containing 10 kDa lysine-fixable Dextran–FITC (1 mg/ml) and fixed 5 min post injection. A set of three cells was analyzed for TR and FITC fluorescence by both FM and CLSM (A). Two injected cells and one noninjected cell were identified by FITC fluorescence (e), indicated by arrows and an arrowhead in the DIC image, respectively (d). The raw FM image was generated by merging TR images recorded across the entire z-stack (a), background-subtracted (b), and further processed by FFT (c). The raw image of merged CLSM images of the entire z-stack is indicated in f. All the TR fluorescence images (a–c, f) are represented as 3-D projections from a tilted image, including a calibration bar with the following pixel minima and maxima: a, 581/2008; b, 33/1088; c: 0/582; f, 0/1252. The number of fluorescent objects of one of the injected cells indicated by the large arrow in d was plotted as a function of pixel intensities in the case of the FFT-processed FM image and the raw and FFT-processed CLSM images (B). The apparent object size is indicated in C. The maximal sum of all the pixel values in an object and the maximal object size were 2751 and 27 in the case of the FM–FFT image, 20 890 and 17 in the case of the CLSM raw image, and 8080 and 11 in the case of the CLSM–FFT image.
below 20% of the maximal values (Figs. 3B and 3C). We conclude that FFT processing of fluorescence images of virus particles across the entire z-stack of fixed cells generates a homogenous population of virus particles with respect to gray values and dimensions.

**Two Waves of Nuclear Transport of Incoming wt Ad in HeLa Cells**

We therefore used FFT-processed FM to determine the subcellular distribution of Ad-associated TR fluorescence in natural infections of human and monkey cells. Ad–TR, and as a parallel control mutant ts1 Ad–TR, was bound to the cell surface in the cold for 60 min and internalized for different times as described earlier (Greber et al., 1997). Defined r.o.i. were indicated by a plasma membrane staining of fixed cells with an anti-PM Ca$^{2+}$ ATPase antibody and anti-rabbit-Alexa 488 and the nuclear stain DAPI. The plasma membrane stain perfectly matched the outline of the cells imaged by Nomarski differential interference contrast optics (data not shown). Using a thresholding function (described under Materials and Methods), we operationally defined the cell periphery as a 3.5-µm-wide ribbon and the nuclear periphery as an 1.8-µm-wide region extending 0.9 µm from both sides of the nuclear periphery (Fig. 4B). The cytoplasm was defined by the total plasma membrane region minus nuclear and perinuclear regions. TR fluorescence was imaged across the entire z-stack as shown in Fig. 4A for wt Ad. Virus fluorescence in a given region was either expressed relative to the total cell-associated virus fluorescence (Figs. 5A and 5D) or expressed as specific fluorescence intensity per region (Figs. 5B and 5E).

At 0 min of entry (when the virus particles are present at the cell surface) the specific peripheral and cytoplasmic fluorescence levels of both wt and ts1 Ad were approximately equal and only slightly elevated in the perinuclear and nuclear regions (Figs. 5B and 5E). This was possibly due to a nonplanar topology of the plasma membrane proximal to the cell surface. The perfusion fixation with a 60-min internalization time was used instead of a slower or longer time, for example, 24 h, because it has been reported that virus particles are less stable in the long term in this cell type, which may result in a decrease in the fluorescence signal (Greber et al., 1997).

**FIG. 4.** Analysis of incoming Ad–TR by FM and FFT processing. Ad–TR was bound to the surface of HeLa cells in the cold (0 min, A, left) or cold-bound and internalized for 90 min (right), followed by FM and image processing as described under Materials and Methods. Virus fluorescence is shown in red. The plasma membrane is visualized by an anti-PM Ca$^{2+}$ ATPase 1 antibody and anti-rabbit-Alexa 488 secondary antibody (green) and the nucleus is indicated by DAPI staining (blue). Based on the plasma membrane and the nuclear regions, we defined r.o.i. (B) where virus-associated fluorescence was quantitated (see Fig. 5).
FIG. 5. Quantification of incoming Ad fluorescence in defined cellular regions of interest. Wt Ad–TR (A–C) or ts1 Ad–TR (D–F) was bound to the surface of HeLa cells and internalized for indicated times. Fluorescence images of virus particles, plasma membrane, and nucleus were acquired and processed as indicated in Fig. 4. Virus-associated fluorescence was determined in r.o.i. and the mean values were expressed as fractions of total cell-associated virus fluorescence (A, D), including SEM (bars). In addition, r.o.i. were plotted as a function of infection time (C, F) and virus-associated fluorescence was normalized to the r.o.i. (B, E). The number of cells analyzed is indicated in C and F. The mean gray values of wt Ad–TR fluorescence across an entire cell varied from 244 260 to 2 430 968 and the cell size was between 53 927 and 70 215 pixels. In the case of ts1 Ad–TR the fluorescence varied from 104 362 to 179 917 and the cell size from 46 523 to 58 140 pixels. n.d. (not determined) indicates regions where the cytoplasmic and the perinuclear regions overlapped.
mal to the nucleus. At 30 min postwarming, ts1 but not wt virus was removed from the cell periphery and enriched in the perinuclear region (Figs. 5B and 5E). At 60 and 90 min p.i., wt Ad levels were significantly reduced at the cell periphery and strongly increased near the nucleus, whereas the ts1 distribution remained unchanged compared to 30 min p.i. These results were in agreement with virus motility data from live cells, demonstrating that wt Ad was transported predominantly to the MTOC, whereas ts1 was moving to and away from the MTOC at similar frequencies and efficiencies between 30 to 90 min p.i. (Suomalainen et al., 1999). At 90 min p.i., the nuclear levels of wt Ad reached about 64% of the total cell-associated virus fluorescence, representing a more than twofold increase compared to 0 min p.i. (Fig. 5A). At 120 min p.i., however, the nuclear fluorescence of wt Ad was reduced to 51%, and the cytoplasmic and peripheral levels were significantly elevated compared to 90 min p.i. ($P = 0.05$). These results suggested a global redistribution of wt Ad toward the cell periphery between 90 and 120 min p.i. This outward flux of virus was nearly reversed at 240 min, resembling the status at 60 min p.i. ($P = 0.01$). As expected, virus fluorescence in the cell periphery and cytoplasmic region were significantly reduced at 90 min of wt Ad infection both in terms of absolute numbers and on a per-region basis ($P = 0.01$). Likewise, the peripheral levels of the endosomal ts1 virus declined rapidly (within 30 min) and the nuclear levels increased somewhat compared to 0 min p.i. Similar results were obtained with two other cell lines, human epitheloid A549 cells and monkey TC7 fibroblasts (data not shown).

Infection of human cells with wt Ad has been reported to trigger actin rearrangements that are in part mediated by the Rho family GTPases rac and cdc42 (Li et al., 1998), known regulators of the actin cytoskeleton (Tapon and Hall, 1997). We investigated whether or not any of the r.o.i. were altered during Ad infection. As indicated in Fig. 5C, the wt Ad infection significantly decreased the cytoplasmic region from 40% of the total cell region at 0 min p.i. to 35-37% at 30-90 min p.i. ($P = 0.01$, Fig. 5C). Concomitantly, the cell periphery increased from 36 to 39-40% ($P = 0.01$). After 120 min p.i. both cytoplasmic and peripheral regions were indistinguishable from the corresponding regions at 0 min p.i. The data demonstrate that wt Ad-infected cells, but not ts1-infected cells, have a tendency to contract between 0 and 90 min p.i. This effect is specific to wt Ad and is not observed with mutant ts1-infected cells, indicating that the experimental procedures are adequate (Fig. 5F).

Altogether, our results reveal two surprising and not-yet described differences between the wt and the ts1 Ad infection. First, nuclear transport of wt but not ts1 virus occurs in two distinct waves, one from 0 to 90 min, followed by a period of virus transport toward the cell periphery, and another at 120 to 240 min p.i. The second surprising result is that the wt Ad infection significantly reduces the apparent cell size between 0 and 90 min p.i. Both of these results may reflect a different signaling potential of wt and ts1 Ad.

**DISCUSSION**

Here we presented a fluorescence microscopy protocol to quantitate fluorescently tagged Ad in cellular r.o.i. at different times of infection. We have utilized electronically controlled fluorescence microscopy combined with a back-illuminated CCD camera to acquire complete sets of optical sections of fluorescent virus particles through infected cells. Individual images were processed by FFT and merged to give a homogenous representation of virus-associated fluorescence without generating or deleting any virus particles. This was confirmed by CLSM analysis of the same sample in the absence of image processing. The signal of a single virus particle is clearly in the lower end of the intensity spectrum, reaching 60 to 300 pixel units in the case of the 12-bit CCD image (4096 gray levels) and 3 to 20 pixel units in the case of the 8-bit CLSM image (256 gray levels). Using a 12-bit (or higher) CCD camera for signal detection is therefore preferable to an 8-bit CLSM chip, since the higher dynamic range of the CCD image significantly increases the resolution of neighboring low intensity pixels and thus enhances the accuracy of background subtraction and quantification procedures. Detection and amplification of low-intensity objects by photomultipliers used in CLSM also enhances background signals, including electronic noise. This problem is documented in Fig. 3, where FM is directly compared to CLSM. The FM image is considerably smoother than the CLSM image. Moreover, quantification of low-intensity CLSM objects, such as fluorescent virus particles, will always require image processing in order to remove cellular autofluorescence (see, for example, Fig. 3A, f). Last, an FM configuration including a sensitive CCD camera is clearly less expensive than a CLSM, particularly if the latter is operated at multiple wavelengths from different laser units or with a cooled photomultiplier to minimize electronic noise.

The procedure described here reliably accounts for the sum of all virus-associated fluorescence across a given cell without the necessity to count individual particles, which is particularly difficult when viruses accumulate, for example, near the nucleus. We expect that this protocol complements live cell analy-
sis, which is typically conducted in single optical sections of flat cell regions, and will provide an alternative to traditional biochemical fractionation procedures and quantitative electron microscopy. Importantly, this protocol is not limited to fluorescence microscopy but can be readily applied to deblur stacks of CLSM images, as indicated in Fig. 3. We must, however, emphasize that the image-processing procedure described here is not applicable to irregularly shaped objects, since we do not take into account the optical properties of the microscope and have not corrected the shape of the objects with an appropriate point-spread function. Consequently, the procedure does not reflect the real fluorescence intensities of the objects. However, the procedure does accurately reproduce the relative fluorescence levels in subcellular regions, since it corrects for local fluorescence quenching by generating a homogenous population of virus objects. We believe that the simplicity of the present protocol to quantify virus-associated fluorescence within a cell will be applicable to various aspects of quantitative cell biology.

In this study we also described the preparation of fully infectious Texas red-labeled virus particles which are exclusively modified in the major coat protein hexon. Hexon-associated fluorescence is ideal for tracing incoming virus particles, since hexon remains associated with the viral genome until the particles reach the nuclear membrane and disassemble at nuclear pore complexes after about 90 min p.i. Here we have concentrated on the analysis of two types of viruses, wt and mutant ts1 Ad, in the peripheral and the nuclear regions of HeLa cells at different times of infection. Both wt and ts1 Ad are known to enter in an integrin-dependent manner with identical kinetics and efficacies (Greber et al., 1996; Wickham et al., 1993). Unlike ts1 virus, which fails to escape from endosomes, wt Ad effectively penetrates the endosomal membrane shortly after endocytosis and is transported as a naked particle in a microtubule- and dynein-dependent manner toward the nuclear membrane (Suomalainen et al., 1999). Using appropriate fluorescent markers for defining the plasma membrane and the nucleus and translating the corresponding images into binary images has allowed us to automate a nonbiased quantification routine of virus fluorescence in the r.o.i. Our results show for the first time that wt virus is slowly cleared from the cell periphery, whereas the endosomal ts1 is rapidly transported from the periphery to a perinuclear compartment. The data suggest that wt Ad may escape from peripheral early endosomes consistent with a mildly acidic pH requirement for membrane rupture. Cytosolic wt virus may be confined to a peripheral region until it is able to utilize the microtubule-based minus-end-directed transport machinery. Once virus engages with this machinery, it is effectively transported to the nucleus, where it is enriched up to 90 min p.i. Ts1 virus, on the other hand, initially moves in endosomes toward the nucleus without delay, presumably in a microtubule-dependent manner, and later, at 30 to 90 min p.i., travels to the cell periphery and the nucleus at nearly equal efficiencies, in agreement with earlier results (Suomalainen et al., 1999).

Our results reveal two novel features of virus entry, a biphasic nuclear transport of wt Ad and a significant cell contraction during the early phase of wt Ad entry. The first nuclear transport phase lasts until 90 min p.i. and is followed by peripherally-directed transport of virus fluorescence from 90 to 120 min. p.i. Whether this transport represents particles that are broken up or not stably bound to nuclear pore complexes is unknown. From 120 to 240 min p.i., a second nucleus-directed transport wave of Ad occurs, leading to a reduction of peripheral virus particles and an enrichment of nuclear particles. The underlying mechanism for this biphasic virus transport is not known. Conceivably, virus-induced cell signaling affects either the attachment or detachment of actin or MT-dependent motors from cargo or, perhaps less likely, regulates motor efficacy. Alternatively, MT-associated proteins (MAPs) may be targets for Ad signaling and thus regulate the frequencies of virus attachment and detachment to MTs. This principle has recently been demonstrated for vesicular transport regulated by the axonal MAP tau (Trinczek et al., 1999).

The second unexpected result of our analysis is a significant cell contraction within the first 90 min of wt Ad infection. Since this effect is transient and does not occur with ts1 Ad, it might be related to wt Ad-induced cell signaling. Whether the small GTPases rac and cdc42, which regulate cell spreading and have been implicated in Ad endocytosis (Li et al., 1998), are involved in cell contraction can now be analyzed. It will be interesting to test if integrins, which are upstream of rac in the Ad entry pathway (Li et al., 1998) are involved in cell contraction. Alternatively, the transient induction of the raf/mitogen-activated kinase pathway by incoming Ad (Bruder and Kovesdi, 1997) could promote cell migration and, perhaps, interfere with cell contraction and spreading, analogously to the plasminogen-dependent activation of the ras pathway in fibrosarcoma cells (Nguyen et al., 1999). Future experiments will address the nature and mechanisms of early cell responses to Ad infections.

With the discovery of an increasing number of primary and secondary receptors for viruses, fluorescent virus particles are expected to become useful for studies of virus uptake, delivery to the replication
site, and also uncoating of the viral genome. For gene therapists, fluorescent virus vectors are important for evaluating vector binding to cells

ex vivo

and perhaps even facilitate an in vivo analysis of vector spread and clearance from different tissues or the site of administration. The latter process is often ill defined and the responsible cells are poorly characterized. Quantitative procedures to measure virus fluorescence may thus improve a rationale design of vectors for gene-based therapies.

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REFERENCES

and plus end-directed motilities are competing processes for nuclear targeting of adenovirus, J. Cell Biol. 144, 657–672.