Laser microbeam ablation of GFP-labeled nuclear organelles in a living cell

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ABSTRACT

Cancer, development, cellular growth and differentiation are governed by gene expression. Recent molecular and cellular advances to visualize and perturb the pathways of transcriptional regulation, nascent RNA processing, and protein trafficking at the single cell level have been developed. More recently, applications utilizing the green fluorescent marker (GFP) from *Aequorea victoria* have facilitated visualization of these molecular events in a living cell. Specifically, we will describe a novel approach to perturb cellular processes by labeling discrete cellular components of interest with GFP and subsequently altering /ablating them with a laser microbeam.

Keywords: laser microbeams, cellular organelles, microablation, green fluorescent protein

1. INTRODUCTION

Acute promyelocytic leukemia (APL) is a subclass of acute myeloid leukemia and is genetically characterized by a t(15:17) chromosomal translocation that generates a fusion between the promyelocyte (*PML*) gene and the retinoic acid receptor α gene (*RARα*) (Kakizuka, *et al.*). The protein products of *PML* and the oncogenic fusion product, *PMLRARα* have distinctively different cellular localization in the normal and leukemic state respectively (Daniel, *et al*, Dyck, *et al*, & Weis, *et al.*). Specifically, in normal cells, PML was found to reside in discrete nuclear bodies termed the PODs (PML Oncogenic Domain). In the leukemogenic process, the structure or body appears to be disrupted with both PML and PMLRARα protein no longer residing in the discrete nuclear bodies but rather giving rise to a microdot pattern homogeneously dispersed throughout the nucleus. Thus, determination of the molecular function of this PML containing body or POD would provide a further understanding of how the leukemia occurs. It would also offer insight into the mechanisms of action that occur and prevent the cell from following its normal differentiation process. To date, while some proteins of unknown function have been found to also colocalize with these bodies, the PODs have not been purified and thus their function remains elusive. Since it is has yet not been possible to create a cell line that does not possess these bodies, we sought to determine their function by performing laser microbeam ablation in a living cell.

2. MATERIALS & METHODS

2.1 Cell culture

HEp2 fibroblasts were obtained from American Type Culture Collection (Rockville, MD) and cultured in DME media (Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (GIBCO) at 37°C in an atmosphere of 7.5% CO_2. Cells were plated on gridded coverslips (Bellco Glass) to facilitate single cell localization during microinjection and imaging 24 hrs after microinjection.

2.2 Microinjection

Monolayer fields of cells were nuclearly microinjected with 50 ng/µl of plasmid DNA diluted in microinjection buffer containing 100 mM potassium chloride and 5 mM sodium phosphate pH 7.4. The plasmid construct contained a fusion between the *PML* gene (Kakizuka, *et al.*) and the green fluorescent protein (*GFP*) positioned at the C-terminus of the *PML* gene (Ogawa, *et al.*). The S65T mutant (Clontech) was used for the GFP because of its greater excitation peak at 495 nm. Cells were microinjected with approximately 1 x 10^{-15} L/cell utilizing an Eppendorf semiautomatic microinjection system and a Zeiss axiophot inverted microscope. Immediately after microinjection of the plasmid DNA, the cells were returned to the incubator.

2.3 Laser microbeam ablation and imaging with the CATS system

The ablation/inactivation of the PML containing nuclear bodies (PODs) was achieved using a highly focused pulsed laser microbeam of a frequency doubled Q-switched Nd:YAG (532 nm) laser. The parameters were as follows: pulse width was 4-6 ns; repetition rate was 10 Hz; average energy was 0.1-0.5 µJ/pulse at the focal plane of the objective; and irradiation time was 15 seconds per POD. Each cell contains between 10 to 20 PODs within its nucleus. The laser beam was delivered through an epi-fluorescence port of a CATS-1 (Confocal Ablation Trapping System) based on a Zeiss Axiovert 135 inverted microscope with a confocal laser scanning system (Zeiss LSM 410) with a 100X 1.3N.A. oil immersed Neofluar objective. Estimation and visualization of imposed damage to the bodies was based on loss of fluorescence of the GFP as assessed by scanning with an Ar-laser (488 nm) of the LSM 410 or direct observation of green fluorescence using standard epi-fluorescence (excitation provided by a 10W HBO lamp light filtered through a 450-490 nm band-pass filter, emission isolated at 510-525 nm). Fluorescence was observed through the eye-pieces or with a color-cooled CCD camera with on-line processing.

2.4 Immunohistochemistry

Cells were fixed in 3.7% formaldehyde/PBS for 5 minutes followed by permeablization with 0.3% TritonX-100 / PBS. Subsequently cells were incubated with an affinity-purified antibody IgG (Dyck, *et al.*) raised to a synthetic peptide corresponding to amino acid 1-14 of the amino terminal end of PML for 1 hour. Cells were then washed and incubated with a secondary antibody (IgG) coupled with Texas Red. After washing, cells were imaged for Texas Red by scanning with an Ar-laser (544 nm).

3. RESULTS

In order to assess the function of a discrete cellular component, we developed a novel approach that combines laser microbeam ablation and GFP labeling of the target in living cells.

3.1 Microbeam ablation

Hep2 cells were microinjected with the PMLGFP expression plasmid. 24 hrs after microinjection, cells expressing PML-GFP were imaged and discrete nuclear bodies or PODs were easily identified (ex488; em 510 - 525 nm) before (Figure 1B) and after (Figure 1C) ablation. All of the PODs within a given cell were pulsed 15 seconds with the second harmonic (532 nm) of an Nd:YAG pulsed-nanosecond laser. Four hours postablation, cells could be located by their coordinates in reference to their location on the alphabetically etched glass coverslip. Cells containing PODs that had been ablated appeared to possess normal morphology (Figure 1C). Furthermore, they maintain their post ablation appearance or "drilled" effect. There were no new PODs synthesized by the cell nor were the damaged structures repaired to the extent that new PMLGFP was not transported there. As a control, fields of cells in a defined location were ablated with the same energy and duration of time and monitored 24 hrs post ablation. These fields of cells appeared normal and continued to divide. We next further extended the duration in which we followed the cells after the initial ablation to 14 hours. By 14 hours those cells that had all of their PML containing nuclear bodies ablated were no longer present by 14 hrs and therefore underwent necrosis more likely apoptosis most likely due to the loss of the nuclear body rather than any effect of the laser since immediately necrosis would be seen by the 4 hr time point post ablation.

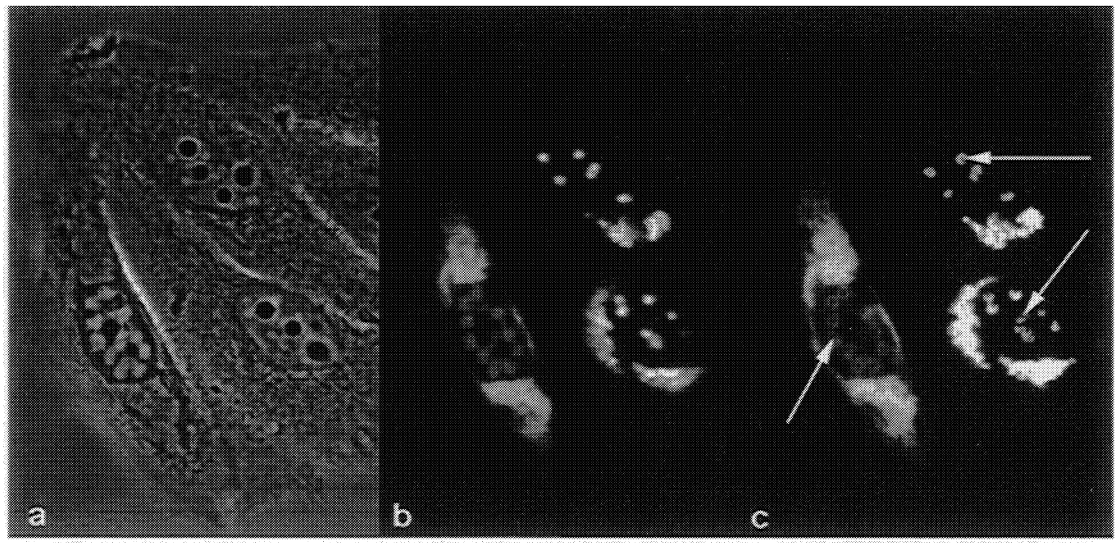

Figure 1. Laser microbeam ablation of PODs. a) Phase contrast micrograph of HEp2 cells b) corresponding fluorescent micrograph of a cluster of living cells expressing PMLGFP fusion protein. Note the labeling of the fluorescent nuclear bodies (PODs). PODs range in size from 0.3 μM to 1.0 μM in diameter. c) the same field of cells after laser microbeam ablation. Loss of POD fluorescence can be seen in the center of the body yielding a "drilled" appearance. Nuclear membrane and nucleoplasm appears to remain intact. All of the PODs were ablated and structurally appear to be absent as assessed by a lack of GFP fluorescence (*e.g.* see arrows). 100X, 1.3N.A.

3.2 Ablation or perturbation of the nuclear body

To determine whether or not the "permanent" ablated appearance (Figure 2A) of the bodies was a structural alteration in the POD or a photobleaching of the green fluorescent protein, we chose to detect PML protein, both endogenous as well as the fusion protein, by indirect immunohistochemistry. To this end, 4 hours after laser microbeam ablation was performed, the cells were imaged for the detection of the green fluorescent protein (PMLGFP) and then subsequently immunostained with an antibody raised against the PML protein as described in Material and Methods. The cells that were ablated possessed the same fluorescent pattern for staining of PML protein as they did for the PMLGFP image. There also were no new POD structures present in these cells. Taken together, we can conclude that laser microbeam ablation appears to be altering the structure of the POD and not simply photobleaching the green fluorescent protein. Furthermore, no new PODs appear to be assembled to subserve the function of the damaged ones. Of course, we can not conclusively demonstrate that endogenous PML is present in the POD and that the epitope recognized by the antibody was altered, thus yielding no antigenic detection. This is highly unlikely, as PMLGFP, despite being continuously present in the cytoplasm of the cell, did not "repair" nor was it redirected to these structures. Future experiments require the examination of the ultrastructure of the damaged PODs by electron microscopy to ultimately demonstrate structural alteration of these nuclear bodies.

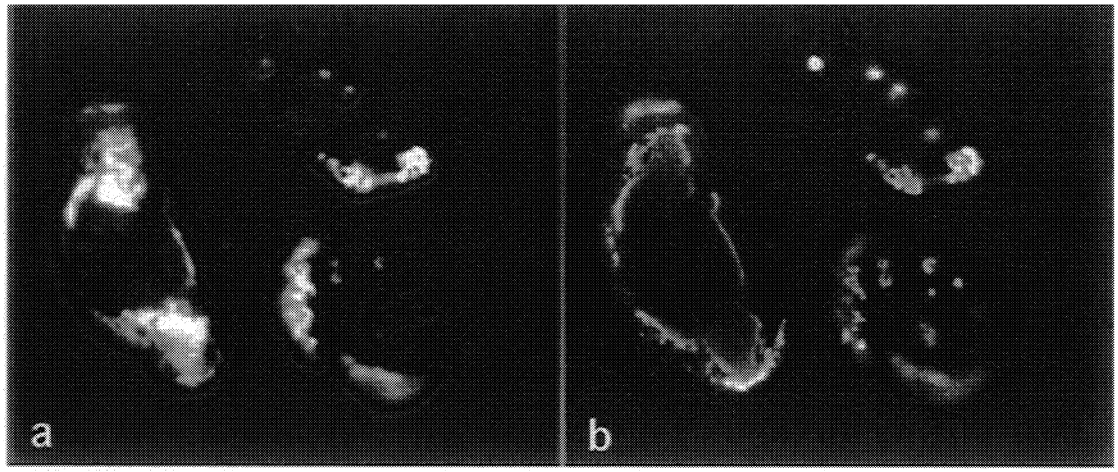

Figure 2. Alteration in the structure of the POD. A) A field of fluorescent HEp2 cells 4 hrs postablation. B) The same field of cells after immunostaining with an affinity-purified antibody to PML (Texas Red). Note that both images correspond with each other suggesting that the PML containing nuclear bodies do not repair themselves with either endogenous PML or PMLGFP or else the bodies would appear "undrilled". 100X, 1.3N.A.

4. CONCLUSIONS

These findings demonstrate the feasibility of laser microbeam ablation of subcellular organelles or components in combination with fluorescent tagging of the structure (GFP), and invite future experiments whereby the function of a given structure within the cell can assessed by removing it and assessing the cell's physiology in its absence.

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