Analysis of Cell-Cycle Profiles in Transfected Cells Using a Membrane-Targeted GFP

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At present, the most commonly used method for determining the cell-cycle profiles (G1, S and G2/M phases) in mammalian cells is to co-transfect the gene(s) of interest with a second plasmid expressing a cell-surface protein that can be identified by immunofluorescence-based cell staining and then analyze the fluorescently stained cells using flow cytometry (9,10). The cluster of differentiation (CD) proteins of T and B lymphocytes are often chosen as the cell-surface markers for this purpose because they are only expressed in T and B cells and, even when expressed at high levels, are not toxic to the common recipient cells used in transfection assays (9,10). However, detection of the cell-surface marker in these transfection assays requires a complicated protocol and reagents. The cells must be detached from tissue culture plates without trypsinization because the cell-surface marker can be destroyed by trypsin. Such treatment, however, makes it difficult to generate a single-cell suspension that is essential for flow cytometry analysis. After detachment from the plates, the cells have to be stained with a specific monoclonal antibody against the transfected cell-surface marker. To detect the signal in flow cytometry analysis, the monoclonal antibody used in the assay needs to be either directly conjugated with fluorescein isothiocyanate (FITC) or detected by FITC-conjugated anti-mouse...
secondary antibodies.

Expression of green fluorescent protein (GFP) can be easily viewed in living and formaldehyde-fixed cells using a fluorescence microscope and a standard fluorescein filter (2,8). Because it does not affect cell function when expressed, GFP becomes an extremely useful co-transfection marker for the gene(s) of interest (8). Nevertheless, as a small soluble protein, GFP is not localized in any specific cellular compartment, and it is easily leached out from cells after fixation and permeabilization with ethanol (GFP Application Notes, p. 23; CLONTECH Laboratories, Palo Alto, CA, USA). Therefore, GFP has not been commonly used as a marker of transfected cells in flow cytometry analysis. To avoid the problem, we made a modification that targets GFP to the cell plasma membrane. The C terminus of enhanced GFP (EGFP) (CLONTECH), which has a single, strong, red-shifted excitation peak at 488 nm, was fused with the last 20 amino acids of c-Ha-Ras, a sequence that provides farnesylation and palmitoylation signals for targeting the Ras protein to the plasma membrane (1,3). The cytomegalovirus (CMV)-driven expression vectors for EGFP-farnesylated (EGFPF) and the parental EGFP were transiently transfected into human HeLa and U2OS cells, and the expression of EGFPF and EGFP was examined using a fluorescence microscope after fixation with formaldehyde (Figure 1). Consistent with previous studies, the green fluorescent signals were detected in both the cytoplasm and nuclei of HeLa and U2OS cells that were transfected with the EGFP construct. By contrast, the green fluorescent signals were detected only in the plasma membrane of HeLa and U2OS cells that were transfected with the EGFPF construct. This result indicated that modification of GFP with a membrane-targeting signal sequence caused the GFP to relocalize to the plasma membrane. Recently, similar results were also

Figure 1. Localization of EGFP and EGFPF in human HeLa and U2OS cells. HeLa and U2OS cells were grown on glass coverslips and transiently transfected with the EGFP and EGFPF plasmids. After fixation with PBS containing 3% formaldehyde, the green fluorescent signals were visualized using a fluorescence microscope. The filter set used was a standard FITC set (Chroma Technology, Brattleboro, VT, USA). The maximum excitation and emission wavelengths for the filter set were 485 nm (range 460–510 nm) and 540 nm (range 515–565 nm).
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obtained by another group using adenoviral gene transfer to express a membrane-targeted GFP in neural cells (7).

Because the expressed EGFPF was localized to the plasma membrane, we next tested whether EGFPF can be used as a co-transfection marker in flow cytometry analysis. It has been well-documented that the D-type cyclin-dependent kinase inhibitor p16ink4A causes Rb-positive cells to arrest in the G1 phase of the cell cycle when it is over-expressed (4–6). We co-transfected a human p16 expression vector under control of a CMV promoter with the EGFPF expression vector at a ratio of 10:1 into the Rb-positive cell line U2OS. As a control, U2OS cells were also co-transfected with a control vector and the EGFPF plasmid at the same ratio. After transfection, cells were washed twice with phosphate-buffered saline (PBS) and incubated with fresh Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS). Forty-eight hours after the removal of DNA precipitates, cells were subjected to flow cytometry analysis. Briefly, the cells were detached from the plates by the standard trypsinization method and then fixed in ice-cold 80% ethanol. Before flow cytometry analysis, the cells were pelleted and then stained in a solution of 40 µg/mL propidium iodide and 80 µg/mL RNase A. Flow cytometry analysis was performed on a FACScan™ (Becton Dickinson Immunocytometry, San Jose, CA, USA). A gate was set to select EGFPF-positive cells with a green fluorescent signal at least 40 times stronger than that in negative cells. As shown in Figure 2, EGFPF-positive cells can be easily detected in both p16 and vector control transfections after the cells have been fixed with ethanol. The cell-cycle profiles in the EGFPF-positive and -negative cells transfected with the control vector and in the EGFPF-negative cells transfected with the p16 plasmid were virtually identical (40% in G1, 45% in S and 15% in G2/M). As expected, the EGFPF-positive cells transfected with the p16 plasmid displayed a significant decrease of S- and G2/M-phase cells and a corresponding increase in G1-phase cells (86% in G1, 8% in S and 6% in G2/M). These results indicated that the membrane-targeted GFP (EGFPF) remained cell-associated even after cells had been trypsinized, fixed and permeabilized with ethanol and therefore can be used as a convenient co-transfection marker for flow cytometry analysis. The EGFPF was shown to be retained in the plasma membrane in trypsinized, ethanol-fixed cells by fluorescence microscopy (data not shown). This EGFPF construct is now routinely used as a co-transfection marker in flow cytometry analysis as well as for cell-sorting and focus assays for gene(s) of interest in our laboratory.

REFERENCES

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Chemiluminescence-Based Detection of Minute Amounts of Apoptotic DNA

Apoptosis describes a series of stereotypical changes that cells undergo during programmed or physiological cell death. It includes characteristic morphological alterations such as cell shrinkage, membrane blebbing and nuclear condensation, which is frequently accompanied by endonuclease-dependent chromatim fragmentation (1). In contrast to necrosis, this particular mode of cell death involves a unique cascade of preprogrammed events that finally results in a stepwise disintegration into cell remnants, which are then efficiently removed by the neighboring cells without evoking inflammation (12).

The internucleosomal cleavage of DNA is a prominent feature of apoptosis, which can be classically visualized by agarose gel electrophoresis and ethidium bromide staining as a discontinuous “ladder” of discrete 185–200-bp multimeric bands (3). Although in some experimental models, if a sufficient amount of DNA is available, apoptotic DNA fragmentation can be easily detected after UV visualization of ethidium bromide or silver staining of gels (7), these methods may not be sensitive enough to reveal the typical oligosomal ladder configuration of apoptosis when only a limited quantity of tissue or cells is available; or, as is frequently the case, only a discrete population of cells undergo apoptosis, and consequently, chromatin fragmentation can be masked by necrotic and/or undegraded DNA (9).

To increase the sensitivity of the method, a variety of enzymatic procedures involving end labeling of DNA fragments using $^{32}$P nucleotides and Klenow polymerase (10), terminal deoxynucleotidyl transferase (5,11) or Taq DNA polymerase (4) have been developed to demonstrate electrophoretic DNA laddering in the presence of low levels of cleaved DNA. Although these isotopic procedures are sensitive and at the same time frequently allow quantitative evaluation of the autoradiographs, among other inconveniences derived from the use of radioactive compounds, they are time-consuming and often give rise to high background signals. A sensitive technique based on Southern blot hybridization with radio-labeled DNA (5) has been recently adapted for the chemiluminescence-based detection of DNA fragments in minute amounts of apoptotic cells (8), but the procedure also bears the inconvenience of being both time-consuming and dependent on a random-primed digoxigenin (DIG-11-dUTP)-labeled probe for each cell type.

To circumvent these problems, we present an alternative approach for the rapid nonisotopic detection of DNA laddering that at the same time allows the quantitative estimation of the well-individualized internucleosomal bands.