[Cell Cycle 4:3, e53-e55, EPUB Ahead of Print: http://www.landesbioscience.com/journals/cc/abstract.php?id=1525; March 2005]; ©2005 Landes Bioscience

Brief Report

Cell Cycle Markers for Live Cell Analyses

Hariharan P. Easwaran[1]
Heinrich Leonhardt[1,2,†]
M. Cristina Cardoso[1,†,*]

[1]Max Delbrück Center for Molecular Medicine; Berlin, Germany

[2]Ludwig Maximilians University Munich; Department of Biology II; Planegg-Martinsried, Germany

†These authors contributed equally to this work.

*Correspondence to: M. Cristina Cardoso; Max Delbrück Center for Molecular Medicine; FVK, Wiltbergstr. 50; 13125 Berlin, Germany; Tel.: +49.30.94172273; Fax: +49.30.94172336; Email: cardoso@mdc-berlin.de

Received 01/05/05; Accepted 01/07/05

This manuscript has been published online, prior to printing for Cell Cycle, Volume 4, Issue 3. Definitive page numbers have not been assigned. The current citation is: Cell Cycle 2005; 4(3):
http://www.landesbioscience.com/journals/cc/abstract.php?id=1525
Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS

cell cycle, DNA replication, S phase marker, G_2 phase marker, PCNA, DNA Ligase I, Dnmt1, live cell microscopy

ABBREVIATIONS

RF	replication foci
GFP	green fluorescent protein
RFP	DsRed1 fluorescent protein
PCNA	proliferating cell nuclear antigen
Dnmt1	DNA methyltransferase 1

ACKNOWLEDGEMENTS

We are indebted to Ingrid Grunewald and Danny Nowak for their excellent technical assistance.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Volkswagenstiftung to H.L. and M.C.C.

ABSTRACT

Many cellular processes are regulated by cell cycle dependent changes in protein dynamics and localization. Studying these changes in vivo requires methods to distinguish the different cell cycle stages. Here we demonstrate the use of DNA Ligase I fused to DsRed1 as an in situ marker to identify S phase and the subsequent transition to G_2 in live cells. Using this marker, we observed changes in the nuclear distribution of Dnmt1 during cell cycle progression. Based on the different nuclear distribution of DNA Ligase I and Dnmt1 in G_2 and G_1, we demonstrate that the combination of both proteins allows the direct discrimination of all cell cycle phases using either immunostainings or fusions with fluorescent proteins. These markers are new tools to directly study cell cycle dependent processes in both, fixed and living cells.

INTRODUCTION

The distribution and dynamics of proteins in living cells can provide important clues as to their function and regulation. The most common and successful method for real-time visualization and tracking of proteins is to use translational fusions with fluorescent proteins.[1-3] Such studies have revolutionized our understanding of dynamic and complex processes in living cells. However, proteins undergo dynamic changes in their subcellular distribution and interactions throughout the cell cycle, which often alter their regulatory or enzymatic activity. The analysis of such cell cycle dependent changes in living cells have been hampered by the lack of suitable markers that allow identification of the various cell cycle stages.

Here we develop and demonstrate the use of a DNA replication factor, DNA Ligase I, fused to the DsRed1 fluorescent protein (RFP-Ligase), as a marker to follow cell cycle progression in living cells. Using RFP-Ligase as a live cell cycle marker, we followed the dynamic distribution of the maintenance DNA methyltransferase Dnmt1 throughout the cell cycle. Based on the characteristic sub-nuclear localization of Dnmt1 at different cell cycle stages, we further propose here the combination of fluorescent protein fusions with Dnmt1 and DNA Ligase I, or immunostaining these endogenous proteins, as a simple strategy to directly identify all cell cycle stages in live or fixed cells.

MATERIALS AND METHODS

Construction of plasmids encoding the fusion proteins. An expression plasmid encoding a translational fusion of Dnmt1 with GFP was made by sub-cloning the KpnI/XmaI fragment of pEMT[4] containing the mouse Dnmt1 ORF into pEGFP-C1 (Clontech) cut with the same enzymes. The RFP-Ligase expression vector was made by sub-cloning the Xho I/Sac II fragment of peGFP-Ligase[5] containing the human DNA Ligase I cDNA into the same sites of pDsRed1-C1 (Clontech). Transcription is driven by the cytomegalovirus immediate-early enhancer-promoter. All plasmid constructs were made using standard cloning techniques.

Cell culture, transfection and Western blot analysis. Mouse C2C12 myoblasts[6] and monkey COS-7 cells (ATCC) were cultured in DMEM supplemented with 20% and 10% FCS, respectively.

C2C12 cells were transfected with plasmid DNA using the calcium phosphate-DNA coprecipitation method followed by a glycerol shock as described before[5] and incubated overnight before immunostaining or live cell microscopy.

COS-7 cells were transfected using Polyfect reagent following the instructions of the manufacturer (Qiagen). After 48–72 hrs of transfection, cells were washed in PBS, harvested and extracted in Laemmli sample buffer (2% SDS, 20% glycerol, 250 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 0.1% bromophenol blue) by boiling at 100°C for 5 min. Proteins were separated by SDS-PAGE and Western blot analysis was done using standard protocols.

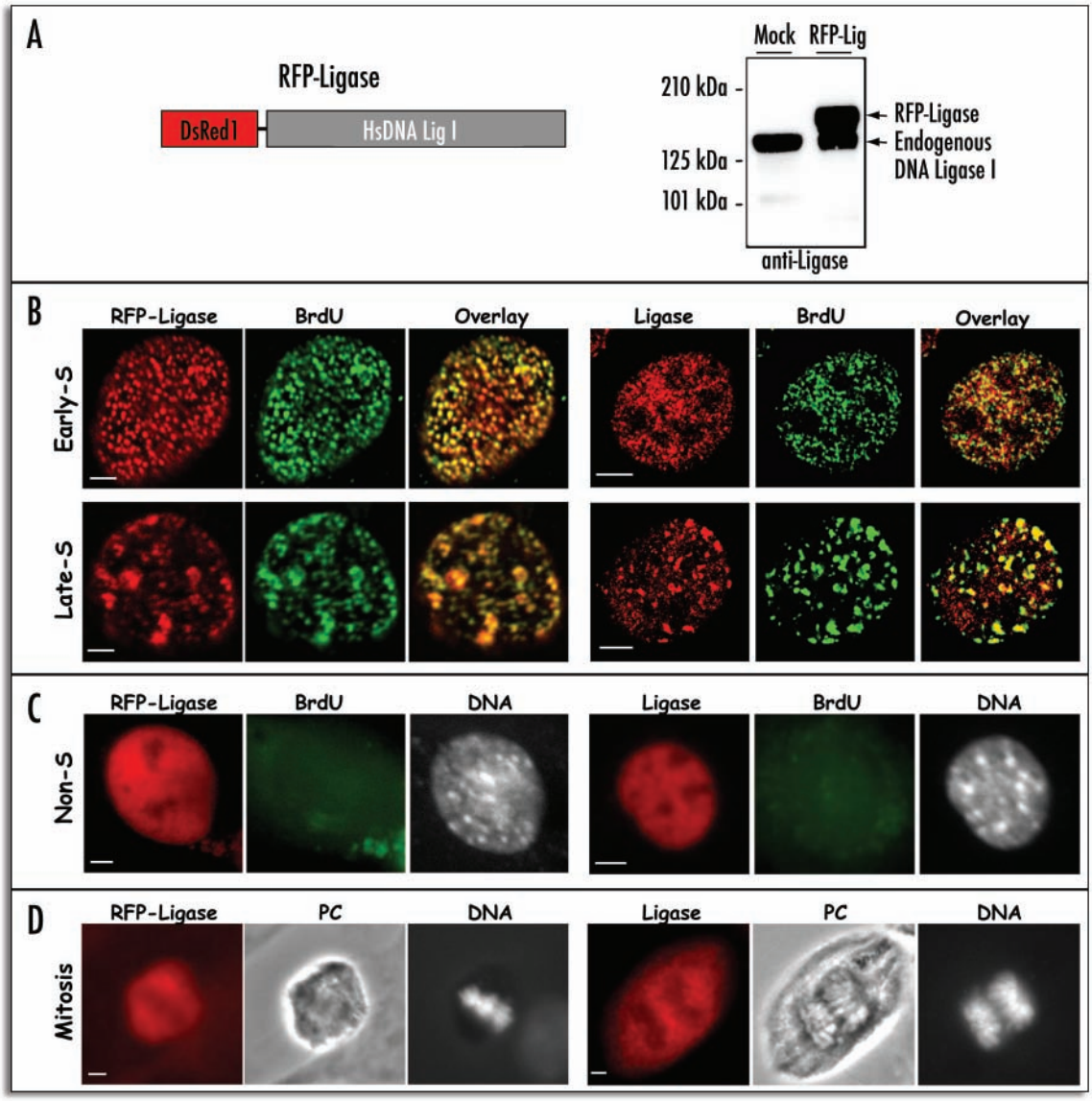

Figure 1. Characterization of a fluorescent S phase marker for live cell analysis. (A) Monkey COS-7 cells were transfected with the RFP-Ligase expression plasmid and, after 48 hrs, the cells were harvested and extracted by boiling in Laemmli sample buffer and analyzed by immunoblotting with an antibody against DNA Ligase I.[5] Mock represents transfection of cells without plasmid DNA. (B-D) RFP-Ligase behaves like the endogenous DNA Ligase I throughout the cell cycle in mammalian cells. On the left, mouse C2C12 myoblast cells were transfected with RFP-Ligase expression plasmid and pulse labeled for 15 minutes with BrdU 24 hrs later, followed by indirect immunostaining for BrdU. On the right, untransfected cells were pulse labeled with BrdU and coimmunostained with antibodies against DNA Ligase I[5] and BrdU. DNA was counterstained with Hoechst 33258. PC stands for phase contrast. Scale bar 5 μm.

BrdU labeling of replication foci and immunofluorescence microscopy. For detection of RF, cells grown on coverslips were incubated in medium with 100 μM BrdU for 15 min (pulse labeling). The cells were then washed two times with PBS and fixed with 3.7% formaldehyde in PBS. Cells were permeabilized with 0.25% Triton-X-100 for 10 min followed by wash with PBS. Nonspecific binding of antibodies was prevented by blocking in 0.2% fish skin gelatin (FSG) for 30 min. Cells were then incubated with mouse monoclonal anti-BrdU antibody (Becton-Dickinson) with or without rabbit anti-DNA Ligase I affinity purified antibody[5] for 1 hr at 37°C. The primary antibodies were diluted in a buffer containing 0.2% FSG, 20U/ml DNase I (Boehringer Mannheim), 0.5 mM β-mercaptoethanol, 0.33 mM $MgCl_2$, 33 mM Tris-Cl pH 8.1. Cells were then washed with 0.1% NP40 in PBS followed by incubation with anti-mouse IgG-FITC and anti-rabbit IgG-TR conjugated secondary antibodies (Jackson Immuno Research) at room temperature. The DNA was counterstained with Hoechst 33258.

Stained cells were analyzed using a Zeiss LSM 510 Meta confocal microscope equipped with a 63x/1.4 NA Plan-Apochromat objective. Ar-laser (488, 514 nm), HeNe-laser 1 (543 nm) and HeNe-laser 2 (633 nm) were used to excite the fluorophores. Hoechst 33258 counterstained nuclei were analyzed on an Axioplan 2 widefield epifluorescence microscope and images acquired with a cooled CCD camera (SensiCam) using Zeiss Axiovision software and appropriate filter sets (Fig. 1C and D).

Live-cell microscopy. Live cell analysis was performed by plating the cells on 40 mm glass coverslips before transfection. One day after transfection, the coverslip was mounted onto the microscope stage and maintained at 37°C using a FCS2 live-cell chamber and temperature controller (Bioptechs) as described before.[7]

For time lapse analysis, images were acquired with a Zeiss LSM 510 Meta confocal microscope using the 488 nm and 543 nm laser lines of an Argon ion laser at low power (1–5%). Four z-sections at 1 μm interval were

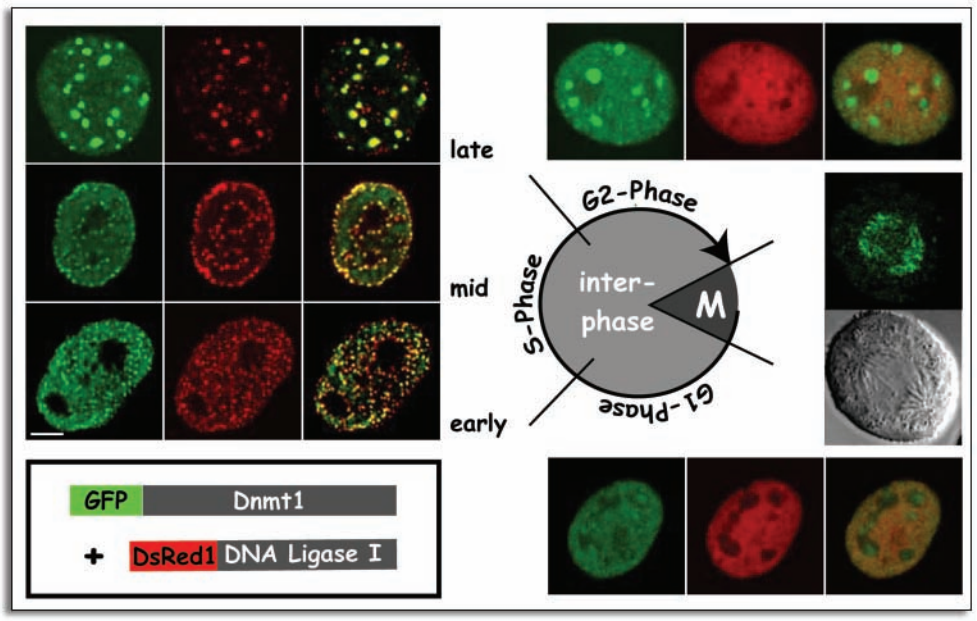

Figure 2. Cell cycle markers for live and fixed cells. The structure of the fluorescent fusion proteins, RFP-Ligase and GFP-Dnmt1, is schematically outlined (bottom left) and the central diagram indicates the respective cell cycle stages from which the images were taken. Mouse C2C12 myoblast cells were transfected with both expression plasmids and live cell microscopy analysis performed one day later. The characteristic distribution of RFP-Ligase (red) makes it possible to identify cells in S phase and to follow cell cycle progression in live cells (see text for details). The combination of GFP-Dnmt1 (green) and RFP-Ligase (red) allows in addition to distinguish between G_1 and G_2 stage in fixed as well as live cells. Colocalization of RFP-Ligase and GFP-Dnmt1 at RF during S phase is observed as yellow in the merged images. During G_2, only GFP-Dnmt1 shows accumulation at centromeric heterochromatin while RFP-Ligase is diffused. During mitosis GFP-Dnmt1 remains bound at condensed chromosomes. In G_1, both RFP-Ligase and GFP- Dnmt1 are diffused (yellow). Therefore, this marker combination makes it possible to directly identify cells in G_1, S and G_2 phase.

imaged every 30–60 min following the cells throughout the cell cycle. After image acquisition, the different z-sections at each time point were aligned manually to correct for movements in the z-plane.

RESULTS AND DISCUSSION

We have previously shown that DNA Ligase I fused to GFP associates with sites of DNA replication (termed replication foci, RF) during S phase.[5] We now fused DNA Ligase I with DsRed1 (RFP-Ligase) for application as a live cell cycle marker in conjunction with proteins fused to GFP. The protein expressed in mammalian cells (COS-7 cells) was checked first by western blot analysis using anti-DNA Ligase I antibodies and showed the expected relative size (Fig. 1A) We then characterized the subnuclear localization of RFP-Ligase in formaldehyde fixed mammalian cells. Mouse myoblast cells (C2C12) were transfected with the RFP-Ligase expression plasmid and showed in S phase cells punctate patterns of RFP-Ligase that colocalize with BrdU labeled replication foci (Fig. 1B). In cells that do not incorporate BrdU (non-S phase cells), RFP-Ligase showed a diffused nuclear distribution and was excluded from the chromatin during mitosis (Fig. 1C and D). This dynamic subnuclear localization pattern of RFP-Ligase mimics endogenous DNA Ligase I (Fig. 1B–D).[5] Moreover, RFP-Ligase, like the endogenous DNA Ligase I, allows further subdivision of early-, mid- and late-S phase based on the typical subnuclear RF patterns (Figs. 1 and 2).[7,8]

We utilized the dynamic subnuclear localization pattern of RFP-Ligase to directly identify each of the cell cycle stages in live cells. Cells in G_2 phase can be identified by following their exit from S phase, which is visible as change from punctate to dispersed nuclear distribution of RFP-Ligase. By following mitotic cells that can easily be distinguished by their typical morphology, we identified cells that enter G_1 phase and show diffused RFP-Ligase. Similar results were obtained using other mouse cell types as well as human cells (data not shown). Thus, using this simple strategy we could unequivocally directly identify in situ all the cell cycle stages (Fig. 2).

We exploited this RFP-Ligase S phase marker to investigate the cell cycle dependent subnuclear distribution of the maintenance DNA methyltransferase (Dnmt1) fused to GFP in live mouse myoblast cells. We found that Dnmt1 associates with sites of DNA replication during S phase as described before.[9] Unlike DNA Ligase I and other replication proteins, however, Dnmt1 is retained preferentially at late replicating centromeric heterochromatin during G_2 and M and is dispersed in the nucleus during G_1.[10] This different distribution in G_2 of GFP-Dnmt1 (focal) and RFP-Ligase (dispersed) can now be used to differentiate between G_1 and G_2 phases in fixed cells as well as in snapshots of live cells without having to follow transitions through S or M phases (Fig. 2). Thus, at any given time, the current cell cycle phase can clearly be deduced from the distribution of these two proteins. This can be done either by expressing fluorescently tagged proteins (as shown here) or in untransfected cells by costaining with antibodies to Dnmt1 and DNA Ligase I (data not shown). Furthermore, other replication factors like PCNA can be used instead of DNA Ligase I since they show a similar subnuclear S phase distribution.[7]

This marker combination provides a precise and direct in situ method to identify all cell cycle stages. The ability to directly identify all the subdivisions of the cell cycle in live (as well as fixed) cells, opens up new experimental approaches to directly study cell cycle dependent processes and related changes in protein dynamics in living cells.

References

1. Lamond AI, Earnshaw WC. Structure and function in the nucleus. Science 1998; 280:547-53.
2. Misteli T. Protein dynamics: Implications for nuclear architecture and gene expression. Science 2001; 291:843-7.
3. Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. Nat Rev Mol Cell Biol 2002; 3:906-18.
4. Czank A, Hauselmann R, Page AW, Leonhardt H, Bestor TH, Schaffner W, Hergersberg M. Expression in mammalian cells of a cloned gene encoding murine DNA methyltransferase. Gene 1991; 109:259-63.
5. Cardoso MC, Joseph C, Rahn HP, Reusch R, Nadal-Ginard B, Leonhardt H. Mapping and use of a sequence that targets DNA ligase I to sites of DNA replication in vivo. J Cell Biol 1997; 139:579-87.
6. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 1977; 270:725-7.
7. Leonhardt H, Rahn HP, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardoso MC. Dynamics of DNA replication factories in living cells. J Cell Biol 2000; 149:271-80.
8. Nakayasu H, Berezney R. Mapping replicational sites in the eucaryotic cell nucleus. J Cell Biol 1989; 108:1-11.
9. Leonhardt H, Page AW, Weier HU, Bestor TH. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 1992; 71:865-73.
10. Easwaran HP, Schermelleh L, Leonhardt H, Cardoso MC. Replication-independent chromatin loading of Dnmt1 during G_2 and M phases. EMBO Rep 2004; 5:1181-6.