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RAD18 and associated proteins are immobilized in nuclear foci in human cells entering S-phase with ultraviolet light-induced damage

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ABSTRACT

Proteins required for translesion DNA synthesis localize in nuclear foci of cells with replication-blocking lesions. The dynamics of this process were examined in human cells with fluorescence-based biophysical techniques. Photobleaching recovery and raster image correlation spectroscopy experiments indicated that involvement in the nuclear foci reduced the movement of RAD18 from diffusion-controlled to virtual immobility. Examination of the mobility of REV1 indicated that it is similarly immobilized when it is observed in nuclear foci. Reducing the level of RAD18 greatly reduced the focal accumulation of REV1 and reduced UV mutagenesis to background frequencies. Fluorescence lifetime measurements indicated that RAD18 and RAD6A or polη only transferred resonance energy when these proteins colocalized in damage-induced nuclear foci, indicating a close physical association only within such foci. Our data support a model in which RAD18 within damage-induced nuclear foci is immobilized and is required for recruitment of Y-family DNA polymerases and subsequent mutagenesis. In the absence of damage these proteins are not physically associated within the nucleoplasm.

1. Introduction

Most mutations induced by carcinogens occur when DNA containing residual damage is replicated during S-phase of the cell cycle. Such lesions perturb the structure of DNA and are likely to block replicative DNA polymerase complexes. Knowledge of fundamental mechanisms involved in the replication of damaged genomes, and of the factors that determine if this process will be error-free or error-prone, is likely to be useful in elucidating the origins of cancer and other human diseases. Originally examined in budding yeast, Lawrence and Hinkle determined that replication-blocking lesions in the template strand can be bypassed by proteins in the RAD6 DNA damage tolerance pathway [1]. Replication of the damaged template is completed by translesion synthesis (TLS) with potentially mutagenic consequences, or by damage avoidance mechanisms mediated by recombination that are largely error-free [2]. These mechanisms are conserved but with additional layers of complexity in higher eukaryotes [3,4]. The ubiquitin conjugating enzyme RAD6 and the associated ligase RAD18 are central to this process. Mutants cannot bypass replication-blocking lesions in the template and are sensitive to many DNA damaging agents. Proliferating cell nuclear antigen (PCNA) ubiquitinated at K164 by the RAD6/18 complex signals TLS [5] and further ubiquitination may signal damage avoidance, although the mechanisms involved in the latter error-free process are poorly understood [3]. Data indicate that PCNA ubiquitinated at K164 has increased affinity for the Y-family polymerases, notably polη and polι [6,7]. Two other members of the Y-family, REV1 and polκ, also contain novel ubiquitin-binding domains [7]. The ubiquitin-binding domain of REV1 is required for functional interaction with PCNA and damage-induced mutagenesis [8], but the catalytic domain of REV1 is dispensable [9]. The principal function of REV1 in TLS is presumably structural since the protein interacts with other Y family polymerases and with REV7 [10]. The latter protein is a subunit of the B-family polymerase ζ [11–13]. In addition to the well-established requirement for RAD18 in the resolution of blocked DNA replication forks, RAD18 has been shown to form nuclear foci in synchronized cells irradiated in G_1, G_1/S, or G_2 phases of the cell cycle [14]. The function of the protein under these circumstances is unknown, but may be related to a physical interaction of RAD18 with RPA coating single stranded regions of DNA [15].

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We examined the intranuclear dynamics of the DNA damage response of a RAD18-eGFP fusion protein in living human cells using a variety of fluorescence dynamics techniques. The fusion protein accumulated in nuclear foci in a small percentage of cells that were undamaged, but the frequency of cells with nuclear foci increased over 10-fold 4 h after UV-irradiation. The increase in the percentage of cells with nuclear foci was strictly dependent on S-phase, since examination of synchronized cells indicated that this increase only occurred in populations that were irradiated at the beginning of S-phase. These foci exhibited a reduction in redistribution after photobleaching, which was quantified by raster image correlation spectroscopy (RICS). Damage-induced focal immobilization of REV1-eGFP was dependent on RAD18. Cells with reduced levels of the latter protein formed foci with a greatly reduced frequency and had slightly enhanced cytotoxic but greatly reduced mutagenic responses to UV. Fluorescence lifetime measurements (FLIM) indicated that RAD18 transferred Förster resonance energy (FRET) to RAD6A or to polη only when the proteins colocalized in damage-induced nuclear foci. This indicates that these proteins are in close physical association only within such foci. These data support a model in which RAD18 is immobilized within damage-induced nuclear foci and is required for subsequent recruitment of proteins required for TLS.

2. Materials and methods

2.1. Cells and cell culture

The primary fibroblast cell strain GM1604 (Coriell Institute) was originally derived from human fetal lung tissue. The telomerase immortalized cells (NF1604) [16] were a generous gift of Dr. Lisa McDaniels (University of Texas Southwestern Medical Center, Dallas) under the terms of MTA 3025 between WGM and Geron Corporation. Cells were kept in exponential growth using published conditions [17].

2.2. Construction of plasmids

The coding sequence of RAD18 was amplified by PCR using the DNA from plasmid pEGLha-hRAD18 [18]. EcoR1 and BamH1 restriction sites were added to the ends of the open reading frame of RAD18. The DNA was ligated into the EGFP-N2 vector (Clontech), such that the fusion protein consists of RAD18 fused with eGFP at the C-terminus of RAD18 (RAD18-eGFP). A PCR product that encoded RAD18 was ligated into pcDNA3 (Invitrogen) in the antisense orientation, for knockdown studies.

For FLIM/FRET studies the coding region for RAD18 was cloned into pAmCyanC1 (Clontech), which expresses CFP fused in-frame with the N-terminus of RAD18. The coding sequences for RAD6A and polη were obtained by reverse-transcription-PCR using total RNA extracted from primary human fibroblasts and cloned into pZsYellow-C1 (Clontech). The latter fusion proteins have YFP fused to the N-terminus of the protein of interest. A plasmid encoding full-length REV1 (base pairs 1-3753) with eGFP fused to the C-terminus of REV1 was constructed as described [19].

All plasmids were sequenced to verify that no mutations had been introduced in the cloning process and that the coding regions were in frame.

2.3. Plasmid transfection and cell synchronization

Electroporation of plasmids was done using the system from Amaxa Biosystems (Amaxa Inc., Gaithersburg, MD) with proprietary reagents and conditions provided by the manufacturer, and as described previously [17,19]. Electroporation reduced cell viability by approximately 10% as determined by clonogenic assays. Following electroporation, cells were cultured for 36 h in complete media to achieve plating and protein expression. Mimosine (0.5 mM final concentration, Sigma, USA) was added to the culture medium for 24 h under low serum conditions (0.1% FBS), after the cells had attached. At this point, the medium was changed, omitting mimosine. To determine the percentage of cells in G_1-, S- or G_2/M-phases of the cell cycle, an aliquot of the cells was trypsinized immediately, 6 h or 12 h after the block was removed. These cells were fixed and stained with propidium iodide. The DNA content was examined with flow cytometry.

2.4. UV-induced cytotoxicity

Cells were plated at cloning density the day before irradiation on 10 cm dishes. The UV source was a Spectroline germicidal lamp, and the flux was measured at 254 nm using a research radiometer fitted with a SED240 photodetector and a W diffuser (International Light, Newburyport, MA, USA). Irradiation was performed as described [17]. The medium was changed 1 week after irradiation, and the cells were stained with crystal violet after 2 weeks.

2.5. Western blot

Cells were collected at ~80% confluence from 15 cm cell culture dishes in lysis solution (10 mM Tris pH 7.4, 1 mM EDTA, 0.1% SDS, and 180 μg/ml PMSF). The solutions were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatants were collected and the protein concentrations were determined. Ten micrograms (RAD18) or 30 μg (PCNA) of whole cell extracts were electrophoresed on 12% SDS-PAGE gels. Following transfer to PVDF filters, the blots were probed with 1:500 mouse anti-RAD18 (Imgenex, San Diego, CA, USA or Abcam, Cambridge, MA, USA) or 1:1000 mouse anti-PCNA (Abcam) and 1:10,000 mouse anti-β actin (Sigma). Horseradish peroxidase (Zymed) was used at a dilution of 1:10,000.

2.6. Determination of the mutagenic effects of UV

A series of independent populations of cells were synchronized by density-inhibition/serum starvation as described [20]. Each population of 1.5×10^6 cells was irradiated 17 h after release from confluence, which corresponds to ~1 h after the onset of S phase. For irradiation, the culture medium was aspirated, and the cells were washed with sterile PBS (pH 7.4). The cells were irradiated as described previously [20] with 8 J/m^2 of $UV_{254\,nm}$, or sham-irradiated. Clonogenic survival was determined, and resistance to thioguanine was examined after an 8-day expression period as described [17].

2.7. Fluorescence recovery after photobleaching (FRAP)

The technology underlying the examination of the redistribution of fluorescently-tagged proteins in living cells depends upon extinguishing the fluorescence of those proteins with an intense laser pulse. This is done in a region of the cell, in this case the nucleus, that is small with respect to the size of the organelle. Proteins that are freely diffusible within the nucleoplasm are detected within the bleached area in several milliseconds. In contrast, constrained proteins do not, such that the bleached area remains so on a time scale of that can be orders of magnitude longer. To conduct these measurements on RAD18-eGFP or REV1-eGFP fusion proteins, cell fluorescence was observed with an inverted Olympus BX-51 microscope illuminated with a filtered Hg arc-lamp. To locally bleach the eGFP, spatially filtered light from an Ar ion laser with a wavelength of 488 nm and a power of 100 mW was focused on the cell. A bleaching pulse of less than 0.5 s in duration was used with a spot size of approximately 1.5 μm in diameter. Following bleaching, the fluorescence signal was monitored continuously within the bleached spot. A PerkinElmer Single Photon Counting Module, Series SPCM-AQR was used. This provides highly sensitive measurements of the fluorescence intensity coming from a 1 μm diameter region of the cell. Fluorescence images of the entire cell can be obtained by scanning the cell with respect to the detector using a piezoelectric drive stage. Post-bleach fluorescent intensity values were normalized to pre-bleach levels, and data from 10 cells of each tested variety were averaged to produce the results presented here.

2.8. Raster image correlation spectroscopy

This technology is based upon the movement of fluorescent proteins into and out of a confocal plane over time. The reason for doing this is to accurately determine diffusion coefficients of fluorescently-labeled proteins within living cells. The confocal image of the cell is scanned with the excitatory laser first in the *x*-direction (microsecond scale), and repeated line-by line (millisecond scale). This process defines raster imaging. One hundred images are recorded sequentially such that the movement of the protein over 2 min is recorded. A rapidly moving protein in the confocal plane will be detected in the *x*-direction and perhaps the *y*-direction, but will not be detected in subsequent images taken over time. The converse will be true of a constrained protein. To conduct these experiments, cells were irradiated with either 10 J/m^2 $UV_{254\,nm}$ or sham irradiated 24 h after transfection. Confocal images were collected 4 h after UV exposure on a XI81 inverted Olympus Fluoview 1000 microscope. A 60×W UPLSAPO objective (1.20 NA) water immersion objective was used to acquire all the images, which were obtained with an Ar ion laser using the 488 nm laser line (Melles Griot, Tokyo, Japan) attenuated to 750 V. A dichroic filter (DM 488/543/633) was used for the laser excitation and for collection of the emission. A bandpass filter (BA 505–605) was selected for eGFP-labeled cells. Images were collected using the Fluoview software with a pixel resolution of 0.03 mm at 256 × 256 pixels with a pixel dwell time of 12.5 μs/pixel. Diffusion coefficients were determined using SimFCS imaging analysis software [21].

2.9. Fluorescence lifetime measurements

Cells were transiently transfected with RAD18-CFP, RAD18-CFP + RAD6A-YFP, or RAD18-CFP + polη-YFP. Cells were irradiated with either 10 J/m^2 $UV_{254\,nm}$ or sham irradiated. Images were collected on a Zeiss Axiovert S100TV microscope 4 h after UV exposure. A 60×W UPLSAPO objective (1.20 NA) water immersion objective was used to acquire all the images. A Ti:Sapphire laser attenuated to 820 nm was