ABSTRACT

We have constructed a human osteogenic sarcoma cell line, U-2 OS/GFP-Gu, that expresses nucleolar RNA helicase RH-II/Gu tagged with green fluorescent protein (GFP). The presence of a GFP tag does not inhibit RNA helicase, RNA folding and ATPase activities of RH-II/Gu protein. The derived cell line responds to cytotoxic agents like the parental cell line U-2 OS. In the presence of either actinomycin D or toyocamycin, the GFP-RH-II/Gu fusion protein translocates from the nucleolus to the nucleoplasm in the same way as the translocation of endogenous RH-II/Gu. There is a need for an efficient, economical and real-time assay to study the mechanisms of drug-induced protein translocation for cytotoxicity screening. Green fluorescent protein (GFP) has been used to tag other proteins used in studies of noninvasive real-time analysis of some cellular processes (7,9).

MATERIALS AND METHODS

Construction of Clones

RH-II/Gu cDNA (12) was polymerase chain reaction (PCR)-amplified using primers BV236 (5'-ATTCCGCG-GCCATGGGATCCGGTGGAGA-AGACCGGT-3'; BamHI site underlined) and BV237 (5'-AACATCATCTCGAGTTCTATATAATCTTCTTCT-3'; XhoI site underlined). The PCR product was digested with BamHI and XhoI, gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA, USA) and subcloned into the BglII and SalI sites of the pEGFP-C1 vector (CLONTECH Laboratories, Palo Alto, CA, USA), resulting in the pEGFP-Gu clone. The pEGFP-Gu DNA used for transfection was prepared using a QIAfilter Plasmid Maxi Kit (Qiagen).

To prepare a His-tagged construct, pEGFP-Gu was digested with NcoI and Xbal. The GFP-Gu cDNA cassette was gel-purified and subcloned into the NcoI and XbaI sites of pProEXHTa (Life Technologies, Gaithersburg, MD, USA), resulting in the pProEX-GFP-Gu clone.

INTRODUCTION

Various drugs cause translocation of specific nucleolar proteins such as nucleophosmin/B23 (13), topoisomerase I (1,5), poly(ADP-ribose) polymerase (6) and RNA helicase RH-II/Gu (10). These protein translocations might be useful in assays to determine the efficacy of cytotoxic agents (1,2,5,13) and detection of drug-resistant cells (3,11). There is a need for an efficient, economical and real-time assay to study the mechanisms of drug-induced protein translocation for cytotoxicity screening. Green fluorescent protein (GFP) has been used to tag other proteins used in studies of noninvasive real-time analysis of some cellular processes (7,9).

The GFP tag does not inhibit the enzymatic activities of RH-II/Gu including RNA unwinding, RNA folding and ATP hydrolysis.

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Cell Culture and Transfection

The human osteogenic sarcoma cell line (U-2 OS 96-HTB) was obtained from ATCC (Rockville, MD, USA). Cells were routinely grown in McCoy’s 5A modified medium containing 10% fetal bovine serum (Sigma Chemical, St. Louis, MO, USA). U-2 OS cells were transfected with the pEGFP-Gu construct using LIPOFECTIN® Reagent (Life Technologies) according to the manufacturer’s instructions. Neomycin-resistant clones were selected using 500 µg/mL GENTICIN® (Life Technologies).

Drug-Induced Protein Translocation

Cells were treated with actinomycin D or toyocamycin as described in

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Figure 1. Translocation of RH-II/Gu induced by actinomycin D. U-2 OS and U-2 OS/GFP-Gu cells were treated with 0.2 µg/mL actinomycin D for 2 h at 37°C. Localization of endogenous RH-II/Gu in untreated (A) or treated (B) U-2 OS cells was determined by indirect immunofluorescence using anti-RH-II/Gu antibodies (12). Localization of GFP-RH-II/Gu fusion protein in untreated (C) or treated (D) U-2 OS/GFP-Gu cells was determined by direct microscopic observation of the cells. The bright fluorescence in Panels A and C corresponds to the nucleoli that were not completely voided of RH-II/Gu (B) or GFP-RH-II/Gu (D) after drug treatment. The weaker fluorescence in Panels B and D corresponds to the nucleoplasm.
Figures 1 and 2. Cell recovery was followed by rinsing out the drug with a fresh medium and incubating at 37°C in 5% CO₂.

Translocation of the endogenous RH-II/Gu was determined by indirect immunofluorescence using anti-RH-II/Gu antibodies as described previously (10). An autoimmune serum containing anti-RH-II/Gu antibodies was obtained from a patient with watermelon stomach disease (12). Translocation of the GFP-tagged RH-II/Gu in stably transfected cells was monitored by placing the tissue culture plate with cells and medium under a Diaphot® TMD inverted fluorescence microscope (Nikon, Melville, NY, USA) with an HBO100 mercury short arc lamp and a filter B-2E with excitation wavelength of 450–490 nm and a barrier filter of 520–560 nm. The maximum emission wavelength of GFP is 507 nm.

Expression and Purification of His₆-Tagged GFP-Gu

A colony of DH5α™ E. coli cells (Life Technologies) transformed with the pProEX-GFP-Gu construct was grown overnight in 50 mL LB medium containing 100 µg/mL ampicillin. One liter of LB medium containing 100 µg/mL ampicillin was inoculated with 20 mL of the overnight culture and allowed to grow at 37°C for 2 h. Expression of the fusion protein was induced with 0.1 mM isopropyl β-d-thiogalactopyranoside (IPTG) for 3 h. Cells were pelleted and sonicated in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine and 10 mM ε-amino caproic acid. The sonicated solution was centrifuged at 20000×g for 30 min at 4°C. The supernatant was passed through a 0.2-µm filter and passed over a TALON™ Metal Affinity Resin (CLONTECH Laboratories) according to the manufacturer’s instructions to purify the His₆-tagged fusion protein. Bound proteins were eluted with 50 mM imidazole in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl. The eluate was dialyzed overnight against 20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM KCl and 10%
glycerol followed by concentration on a 30-kDa NANOSEP® Filter Unit (Pall Filtron, Northborough, MA, USA).

**Enzyme Assays**

The purified His$_6$-tagged GFP-Gu fusion protein was assayed for RNA helicase, RNA folding and ATPase activities according to the previously described procedures (8,12).

**RESULTS AND DISCUSSION**

Treatment of the U-2 OS parental cell line with 0.2 µg/mL actinomycin D for 2 h resulted in translocation of the endogenous RH-II/Gu to the nucleoplasm as shown by indirect immunofluorescence staining of fixed cells using antibodies against RH-II/Gu (Figure 1, A and B), as previously reported in MCF-7 breast cancer cells (10). Under these conditions, complete nucleolar exclusion of RH-II/Gu was not observed as shown by residual nucleolar fluorescence (Figure 1B). Stable transfection of U-2 OS cells with the pEGFP-Gu construct produced a subline, U-2 OS/GFP-Gu, that expresses RH-II/Gu protein tagged with GFP at its amino terminus; the fusion protein localized to the nucleoli, showing that the presence of GFP did not affect the proper cellular localization of the recombinant RH-II/Gu. Treatment of the derived cell line U-2 OS/GFP-Gu with 0.2 µg/mL actinomycin D for 2 h likewise resulted in translocation of the GFP-RH-II/Gu fusion protein from the nucleolus to the nucleoplasm (Figure 1, C and D), albeit less efficiently compared to the translocation of the native RH-II/Gu, which may be because of overexpression of GFP-RH-II/Gu. A higher drug dose is probably necessary to more efficiently cause translocation of both the endogenous RH-II/Gu and exogenous GFP-RH-II/Gu in U-2 OS/GFP-Gu cells. This assumption, however, does not negate the possibility of a subtle biological difference between the two forms of RH-II/Gu protein. The images in Figure 1 (C and D) were obtained by direct observation of live U-2 OS/GFP-Gu cells under the microscope. The GFP portion of the GFP-RH-II/Gu fusion protein fluoresces when excited with 450–490 nm light.

We previously reported that the toyocamycin-induced translocation of RH-II/Gu in MCF-7 cells is reversed by removing the drug from the medium (10). To determine if translocation of GFP-RH-II/Gu was also reversible, U-2 OS/GFP-Gu cells were treated with 50 µM toyocamycin for 2 h. Toyocamycin, like actinomycin D, caused translocation of GFP-RH-II/Gu from the nucleolus to the nucleoplasm (Figure 2, A and B). The drug-containing medium was then replaced with a fresh medium, and the cellular localization of GFP-RH-II/Gu was monitored. Relocalization of GFP-RH-II/Gu to the nucleoli was observed 30 min after fresh medium replaced the drug-containing medium (Figure 2C). The results show that...
both endogenous RH-II/Gu and the recombinant GFP-RH-II/Gu fusion protein respond in a similar manner to cytotoxic agents (10). RH-II/Gu was previously reported to unwind double-stranded RNA in the 5’ to 3’ direction (helicase), to introduce secondary structures into a single-stranded RNA (folding) and to hydrolyze ATP (ATPase) (8). Bacterially expressed RH-II/Gu tagged with glutathione S-transferase (GST) at the amino terminus possesses similar enzymatic activities (12). To prove that the GFP tag does not alter the functions of RH-II/Gu, the GFP-RH-II/Gu fusion protein was tagged with His6, to facilitate purification. Figure 3 shows that the GFP-RH-II/Gu protein has RNA helicase (Figure 3A), RNA folding (Figure 3B) and ATPase (Figure 3C) activities. The results also imply that the amino terminus of the RH-II/Gu protein is probably not critical for its enzymatic activities because tagging of this region did not affect the functions of RH-II/Gu.

The localization of GFP-RH-II/Gu to the nucleolus, its drug-induced translocation to the nucleoplasm and its three enzymatic activities indicate that the GFP-RH-II/Gu behaves similarly to the endogenous RH-II/Gu protein. The cell line developed in this study can be used in a system to study the efficacy of various cytotoxic agents or to rapidly compare the efficiencies of structurally related cytotoxic agents whose actions cause translocation of RH-II/Gu. The cells can be observed under normal growing conditions, the progression of protein translocation is easily monitored, and, together with imaging analysis, kinetic parameters are easily obtained. Quantitative analysis of GFP-RH-II/Gu distribution within the nucleus can be determined by imaging analysis as described previously by Chan et al. (4) for nucleolar protein B23 translocation. Our derived cell line can also be useful in studies on the distribution of RH-II/Gu during the cell cycle and, more specifically, during the formation of the nucleolus. The disadvantages associated with cell fixation and immunostaining are circumvented, and results are seen more rapidly.

The system described in this report can be applied to other cancer cell lines that can be stably transfected with the pEGFP-RH-II/Gu construct. Such derived cell lines will be useful in the studies of drug resistances based on the translocation of GFP-RH-II/Gu fusion protein monitored in vivo and in real time.

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REFERENCES


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