Overexpression of Human DNA Topoisomerase II α by Fusion to Enhanced Green Fluorescent Protein

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Yin-Yuan Mo, Keith A. Ameiss and William T. Beck

University of Illinois at Chicago, Chicago, IL, USA

ABSTRACT

DNA topoisomerase (topo) II α is a major target for many anticancer agents. However, progress towards understanding how these agents interact with this enzyme in human cells and how resistance to these agents arises is greatly impeded by difficulties in expressing this gene. Here, we report on achieving a high level of expression of a full-length human topo II α gene in human cells. We started with the topo II α cDNA driven by a strong cytomegalovirus promoter and transiently transfected HeLa cells. Although topo II α mRNA was consistently detected in transfected cells, no exogenous topo II α protein was detected. By contrast, when the same cDNA was fused to an enhanced green fluorescent protein (EGFP), we detected a high level of expression at both mRNA and protein levels. The exogenous topo II α was localized to cell nuclei as expected, indicating that the fusion protein is properly folded. Furthermore, overexpression of the EGFP-topo II α fusion protein increased the sensitivity of the transfected cells to teniposide, suggesting that it functions as the endogenous counterpart. Thus, in addition to being used as a gene tag, the GFP fusion approach may be generally applicable for expressing genes, such as topo II α , that are difficult to express by conventional methods.

INTRODUCTION

DNA topoisomerase (topo) II is an essential enzyme that regulates DNA topology for DNA replication and transcription during cell growth and cell cycle progression (8). In mammalian cells, although two types of topo II exist, α and β (5,10), topo II α seems to be the predominant isoenzyme in most tissues. Its clinical importance comes from findings that this enzyme is a major target for many anticancer drugs (7). Although much information about the physiological and biochemical aspects of the enzyme has been accumulated from studies of the yeast and *Drosophila* enzyme, little is known about regulation and expression of the gene for human topo II α in mammalian systems. This unfamiliarity has been attributed to the difficulty in expressing the topo II α gene in mammalian systems. For example, several mutations in human topo II α have been identified in drug-resistant cell lines (9), but testing whether any of these mutations confer drug resistance has not been possible in mammalian systems.

Human topo II α is 170-kDa nuclear protein consisting of 1530 amino acids (10). Although some success has been reported in yeast systems (4), as with many other large proteins, expression of this gene in mammalian cells has been a great challenge. While the expression of human topo II α in mammalian cells has been reported (1), the results were difficult to interpret because the exogenous gene product was indistinguishable from the endogenous

counterpart. To express human topo II α in human cells, we have tried a variety of approaches, including use of constitutive strong, weak or inducible promoters (e.g., cytomegalovirus [CMV], Rous sarcoma virus [RSV] and tetracycline, respectively) without success. All these efforts only led to expression of this gene at the transcriptional level, and no exogenous topo II α was detected by Western blot using antibody specific to the exogenous protein (Y.-Y. Mo and W.T. Beck, unpublished results). However, while we were examining expression of the C-terminal region of topo II α for its subcellular localization, we fused the C terminus of the gene to the enhanced green fluorescent protein (EGFP) and found very high expression of the fusion protein. We subsequently examined the possibility of expressing the full-length human topo II α . By using the EGFP fusion, we were able to detect high levels of exogenous topo II expression at both the mRNA and protein levels in mammalian cells. This protein localized to the cell nucleus and appeared to be catalytically active, because it conferred sensitivity to the specific inhibitor of topoisomerase II, teniposide (VM-26).

MATERIALS AND METHODS

Construction of Expression Plasmids

To label human topo II α , we introduced the RGS-6 $\times$ His sequence from a QIAexpressTM pQE high level bacterial

expression vector (Qiagen, Chatsworth, CA, USA) at *KpnI* and *BamHI* sites of pCDNA3 (Invitrogen, Carlsbad, CA, USA) to make an N-terminal tag (pCDNA3-His). This was done so that this fragment could be released by digestion with these two enzymes. For efficient translation of the protein, the Kozak sequence (6) was introduced in the front of the RGS-6×His tag (Figure 1). To facilitate the subcloning of topo II α cDNA into pCDNA3-His, a *BamHI* site was introduced at the N terminus of topo II α cDNA by polymerase chain reaction (PCR) amplification, eliminating the first methionine (start codon) of topo II α . Similarly, the restriction sites *SacII* and *NotI* were introduced at the C terminus after the stop codon. Primers for the N-terminal sequence were: Ila-B1 (sense) 5'-GGATCCGAAGT-GTCACCATTCGAG-3' and Ila-3.1 (antisense) 5'-GGTGGATCCAGCA-ATATCAT-3'; primers for the C terminus were Ila-C1A (sense) 5'-GTGACAGTGAAGAAGACAGCAG-3' and Ila-C18.1 (antisense) 5'-GCGG-CCGCGGTAAAACAGATCATCTT-CATC-3'. Sequences of the PCR products were verified by DNA sequencing.

Plasmid pT14 was constructed by cloning the full-length topo II α cDNA (10) in pCDNA3-His unidirectionally at *BamHI* and *NotI* sites. To construct pT104, pT14 was completely digested with *SacII* but partially digested with *HindIII*, and the full-length topo II α cDNA plus RGS-6×His was fused in-frame to pEGFP-C3 (CLONTECH Laboratories, Palo Alto, CA, USA) at *HindIII* and *SacII* sites. Although this construct contains an internal start codon (ATG), the internal translational product was not detectable.

Transfection, RT-PCR, Immunoblotting and Fluorescent Microscopy

Expression plasmids were introduced into HeLa cells by the calcium phosphate method using 20 μ g DNA per culture dish (100 mm in diameter). Transfection efficiency was monitored by co-transfection of pT14 with pEGFP-C3 or by pT104 alone. Cells were harvested 16 h posttransfection for either RNA isolation or protein extraction. Total RNA was isolated by using TRIzol[®] Reagent (Life Technolo-

gies, Gaithersburg, MD, USA) per the manufacturer's protocol. Contaminating DNA in the RNA samples was removed by treatment with RNase-free DNase before reverse transcription (RT). Primers used to detect exogenous topo II α by RT-PCR were: T1 (sense), 5'-GGTACCGATACCATGCGGGGT-TCT-3' (Figure 1), derived from the vector sequence and T2 (antisense) 5'-TAGCCATTTCGACCACCTGTAC-3', derived from the topo II α sequences. This set of primers allows us to amplify only the exogenous gene (ca. 500 bp) at the N terminus. For Western blotting, total proteins were extracted by lysing cells in dishes with 1× sample buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], and 10% glycerol), and the protein concentration was measured by a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Approximately 50 μ g of proteins were loaded in each lane of a 7% SDS polyacrylamide gel. To detect exogenous proteins, we used anti-RGS-His antibody (Qiagen) and GFP antibody (CLONTECH), respectively; antibody 284 (2) was used to detect both endogenous and exogenous topo II α .

Figure 1. Constructs of human topo II α used in transfection experiments. Primers used for amplifying the N- or C-terminal regions of topo II α by RT-PCR are shown as arrows. Restriction sites (*Eco0109I* and *EcoRI*) were used for convenient cloning of PCR products. RGS-6×His sequence is blocked. As indicated, both constructs use the same CMV promoter (Pcmv), but pT104 carries EGFP fused to topo II α . The drawing is not in scale.

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Signals were visualized with ECLTM Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Subcellular localization of the fusion protein in living cells was monitored under an Axioskop[®] fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) using a filter with maximum excitation at 480 nm and maximum emission at 520 nm.

Growth Inhibition Assay

HeLa cells were transiently transfected with topo II α constructs and seeded into 12-well plates at approximately 0.2 million cells per well. After 24 h of treatment with VM-26 at various concentrations, cells were trypsinized, and cell numbers were determined by using the Coulter[®] Counter (Beckman-Coulter, Miami, FL, USA).

RESULTS AND DISCUSSION

Because it is a large protein, topo II α is difficult to express exogenously. A general consensus view is that expression of exogenous topo II α is cytotoxic and that it is strictly regulated through the cell cycle (3). However, transient expression is presumably not affected by these factors. Therefore, we sought to express topo II α in transiently transfected HeLa cells. To distinguish exogenous topo II α from its endogenous counterpart, we tagged topo II α with RGS-6 $\times$ His at the N terminus (pT14; see Figure 1); an antibody against this tag is commercially available. When pT14 was introduced into HeLa cells, we consistently detected exogenous topo II α mRNA by RT-PCR using a set of primers that specifically amplify the 6 $\times$ His-tagged topo II α . Figure 2A shows an example of such RT-PCRs. This approximately 500-bp fragment specifically came from expression of the exogenous topo II α since no product was seen in PCR using the same RNA sample without RT. Similarly, we saw no products with RNA isolated from cells transfected with vector alone (data not shown). However, no exogenous topo II α protein was detected by anti-His antibody (Figure 2B). Even for some deletion constructs, we detected no expression of exogenous topo II α

protein (data not shown).

By contrast, when the same fragments were fused to EGFP, we found that they were expressed well (data not shown), indicating that EGFP appears to enhance expression of the exogenous gene at the protein level. Consequently, we cloned the full-length topo II α cDNA into pEGFP-C3 (pT104; see Figure 1). Figure 2A shows that, as with pT14, RT-PCR detected comparable exogenous topo II α mRNA from pT104-transfected cells. Figure 2B

shows a 200-kDa band that was detected by Western blotting combined with use of anti-6 $\times$ His antibody. This band is predicted to be the exogenous topo II α , i.e., topo II α (170 kDa) plus EGFP (27 kDa). To confirm this prediction, we stripped the membrane and re-probed with topo II α -specific antibody 284. As expected, antibody 284 reacted with only the 170-kDa band, corresponding with the endogenous topo II α , in pT14-transfected cells. By contrast, two bands (170 and 200 kDa)

Figure 2. Transient expression of topo II α in HeLa cells. (A) Expression of exogenous topo II α mRNA as detected by RT-PCR. An equivalent of 10 ng of total RNA were used for RT-PCR to generate the products seen in each lane. RT, reverse transcription; M, 100-bp DNA markers. (B) Detection of expressed exogenous topo II α protein by Western blot using antibodies specific to topo II α , RGS-6 $\times$ His or GFP as indicated on the left.

Figure 3. Nuclear localization of EGFP-topo II α fusion protein by fluorescent microscopy. HeLa cells were grown overnight on cover slips in culture dishes, followed by transfection with either pT14 plus pEGFP-C3 (A) or pT104 alone (B). Sixteen hours after transfection, cover slips were transferred to glass slides, and expression of EGFP was monitored by fluorescent microscopy. Notice nuclear localization of the EGFP-topo II α fusion protein in Panel B.

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were detected by this antibody in pT104-transfected cells (Figure 2B, middle panel). To further verify that the 200-kDa protein is the EGFP-topo II α fusion protein, an anti-GFP antibody was used to probe the membrane that was stripped for a second time. As with the anti-RGS-6 $\times$ His antibody, the antibody against GFP reacted with only the 200-kDa band but not the 170-kDa

band (Figure 2), suggesting that the 200-kDa band is the exogenous EGFP-topo II α and that the signals from the previous blot were completely removed.

To determine whether low transcription activity leads to undetectable expression of pT14, we used the same set of primers that only amplify the exogenous topo II α cDNA. Semiquantitative

RT-PCR suggested that about same amount of exogenous topo II α mRNA was made for both pT14 and pT104 (data not shown). To exclude the possibility that lack of expression of pT14 could be due to a low transfection efficiency, we determined transfection efficiency by co-transfecting pT14 with pEGFP-C3, because pT14 does not have a monitoring marker. By contrast, the pT104 has the EGFP marker to use to determine transfection efficiency with this construct alone. Average transfection efficiency calculated from more than 10 independent experiments was about 25%. In fact, the efficiency for pT14 was slightly higher than that for pT104. Figure 3 shows expression of these markers following transfection into HeLa cells. As expected, the EGFP-topo II α fusion protein was localized to the cell nucleus, suggesting that the protein is folded properly. Finally, we tested the functionality of the EGFP-topo II α protein by growth inhibition assay. Overexpression of the EGFP-topo II α increases the cell's sensitivity to the topo II inhibitor VM-26 (Figure 4). Inhibitory concentration (IC)₅₀ for the control was 4.6 μ M, whereas IC₅₀ for pT104-transfected cells was 0.9 μ M, suggesting that this fusion protein functions as the endoge-

Figure 4. Effect of expression of the topo II α on sensitivity of cells to VM-26. HeLa cells were transfected with pT14 or pT104 and then treated with VM-26 at the indicated concentrations for 24 h. Data are the means ($\pm$ standard deviation) of three independent experiments.

nous counterpart. Therefore, we demonstrated here that GFP is a valuable tool not only for gene tagging, but also for enhancing the expression of such difficult genes as topo II α .

Achieving overexpression of some genes is often challenging and thus hinders studies of the gene products' physiological functions. Our results suggest that some of these genes might be able to be expressed using this GFP fusion approach. Indeed, our preliminary results with overexpression of topo II β using the same approach seem to support this notion (Y.-Y. Mo and W.T. Beck, unpublished data). The reason for GFP fusion enhancement of topo II α expression is not clear at this point. Since topo II α mRNA levels for both constructs are about the same, it is possible that EGFP either increases translation efficiency or facilitates protein folding, thus stabilizing the protein and preventing degradation. Because both constructs carry the same Kozak sequence in front of the translation start codon (ATG), they should be translated at the same efficiency. If this is the case, EGFP might stabilize the overexpressed topo II α gene product.

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Address correspondence to:

Dr. William T. Beck
Division of Developmental Therapeutics
Cancer Center (M/C 569)
University of Illinois at Chicago
Chicago, IL 60607, USA
Internet: wtbeck@uic.edu