Human topoisomerase IIα nuclear export is mediated by two CRM-1-dependent nuclear export signals

Joel G. Turner[1,*], Roxanne Engel[1,*], Jennifer A. Derderian[1], Richard Jove[2] and Daniel M. Sullivan[1,‡]

[1]Experimental Therapeutics and [2]Molecular Oncology Programs, Departments of Interdisciplinary Oncology and Biochemistry and Molecular Biology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA

*These authors contributed equally to this work

‡Author for correspondence (e-mail: sullivad@moffitt.usf.edu)

Accepted 9 February 2004
Journal of Cell Science 117, 3061-3071 Published by The Company of Biologists 2004
doi:10.1242/jcs.01147

Summary

Resistance to chemotherapeutic drugs is a major obstacle in the treatment of leukemia and multiple myeloma. We have previously found that myeloma and leukemic cells in transition from low-density log phase conditions to high-density plateau phase conditions export substantial amounts of endogenous topoisomerase II alpha from the nucleus to the cytoplasm. In order for topoisomerase-targeted chemotherapy to function, the topoisomerase target must have access to the nuclear DNA. Therefore, the nuclear export of topoisomerase II alpha may contribute to drug resistance, and defining this mechanism may lead to methods to preclude this avenue of resistance. We have identified nuclear export signals for topoisomerase II alpha at amino acids 1017-1028 and 1054-1066, using FITC-labeled BSA-export signal peptide conjugates microinjected into the nuclei of HeLa cells. Functional confirmation of both signals (1017-1028 and 1054-1066) was provided by transfection of human myeloma cells with plasmids containing the gene for a full-length human FLAG-topoisomerase fusion protein, mutated at hydrophobic amino acid residues in the export signals. Of the six putative export signals tested, the two sites above were found to induce export into the cytoplasm. Export by both signals was blocked by treatment of the cells with leptomycin B, indicating that a CRM-1-dependent pathway mediates export. Site-directed mutagenesis of two central hydrophobic residues in either export signal in full-length human topoisomerase blocked export of recombinant FLAG-topoisomerase II alpha, indicating that both signals may be required for export. Interestingly, this pair of nuclear export signals (1017-1028 and 1054-1066) also defines a dimerization domain of the topoisomerase II alpha molecule.

Key words: Topoisomerase II alpha (topo IIα), Nuclear export signal (NES), CRM-1, Site-directed mutagenesis, Microinjection

Introduction

Recent investigations have elucidated several molecular pathways for the nuclear import and export of proteins (Kau and Silver, 2003; Weis, 2003) across transport passageways or nuclear pore complexes (NPCs) (Dreger, 2003). The NPC is a large (125 MDa) multimeric protein structure that perforates the nuclear envelope and channels proteins greater than 60 kDa into or out of the nucleus. The constituents of the NPC have been described in yeast (Rout et al., 2000) and mammalian cells (Cronshaw et al., 2002). Proteins targeted for receptor-mediated transport across the NPC must either contain a nuclear localization signal (NLS) or a nuclear export signal (NES). Protein NLS are typically short clusters of basic amino acids, often preceded by an acidic amino acid or proline residue. However, a NLS may also consist of bipartite clusters of basic amino acids separated by a spacer region of approximately ten amino acids, often flanked by a neutral or acidic amino acid. Previously described NLSs are annotated in SWISS-PROT (Bairoch and Apweiler, 2000) and PIR (Wu et al., 2002), and can be retrieved at the NLS database located at the Predict NLS server (Cokol et al., 2000). Protein NES are hydrophobic rich sequences that have a characteristic spacing of leucine, isoleucine, valine, and/or phenylalanine. To date, approximately 75 experimentally validated protein-NES have been identified and compiled in the NESbase version 1.0 database (La Cour et al., 2003).

In general, protein import occurs when the transport receptors, importin-α and importin-β, form a complex with the protein-NLS and escort the protein cargo across the NPC into the nucleus (Yoneda et al., 1999). The protein cargo is released into the nucleus when importin-α binds Ran-GTP. Protein export occurs when Ran-GTP and the nuclear export receptor, CRM-1, bind to a protein bearing a NES and transports this protein cargo into the cytosol (Fukuda et al., 1997; Fornerod et al., 1997; Ossareh-Nazari et al., 1997). The protein cargo is released into the cytoplasm when Ran-GTP is hydrolyzed by Rna1p, a small GTPase-activating protein. Ran-GDP is then transported back into the nucleus by a small protein called Ntf2 (Ribbeck et al., 1998). A guanine nucleotide exchange factor then swaps out Ran-GDP for GTP. In this manner, continued nuclear-cytoplasmic shuttling occurs by maintaining a gradient of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm (Gorlich, 1998).

DNA topoisomerases (topo) are nuclear enzymes that maintain the topology of DNA during cellular processes such as DNA replication, transcription and genetic recombination (reviewed by Wang, 2002). Different topo enzymes have been identified in humans that are distinguished by differences in

their gene locus, molecular mass, catalytic requirements, mechanism of catalysis, and biological roles. Type I enzymes (topo I) are active as monomers and catalyze transient single strand DNA breaks without ATP hydrolysis. The amino-terminal region of topo I has an important role in regulating the cellular localization of topo I because it contains the nuclear localization signal and nucleolin binding site (Mo et al., 2000). Type II enzymes include topo IIα (170 kDa) and topo IIβ (180 kDa). The type II enzymes form dimers and couple double strand DNA breaks with ATP hydrolysis. Topo IIα is targeted to the nucleus via a bipartite NLS located in the carboxyl terminus, whereas topo IIβ contains two NLS comparable to the region in topo IIα and a third weaker NLS (Cowell et al., 1998; Mirski et al., 1999).

DNA topoisomerases are also the principal targets of commonly used chemotherapeutic agents, including topotecan, doxorubicin, CPT-11 and etoposide. Generally, topo poisons convert the enzyme into a lethal DNA damaging agent, such that the more active enzyme that is present in the nucleus the more effective the poison (reviewed by Engel et al., 2003). Protein degradation and altered subcellular localization of topo have been postulated as two potential mechanisms that contribute to cellular drug resistance by attenuating the amount of drug target in the nucleus (Valkov et al., 1997; Valkov et al., 2000; Sullivan et al., 1987; Kang and Chung, 2002; Engel et al., 2004). For example, adhesion of human myelomonocytoid U937 cells to fibronectin by α1 integrin protects cells against mitoxantrone and etoposide-mediated DNA damage, and is accompanied by an altered sub-nuclear relocalization of topo IIβ to the nucleolus (Hazelhurst et al., 2001). Thus, changes in the nuclear localization or binding properties of the nuclear pool of topo IIβ protein may have a role in cellular drug resistance to etoposide and mitoxantrone in these cells. Furthermore, several cell lines that have been selected for resistance to topo poisons have been characterized for mechanisms of cellular drug resistance and are often shown to express a truncated topo IIα enzyme that has lost its C-terminal NLS so that it remains in the cytoplasm (Wessel et al., 1997; Harker et al., 1995). A truncated topo IIβ has also been reported to associate, in vivo, with the mtDNA genome in bovine mitochondria (Low et al., 2003).

We have previously reported that topo IIα resides in the cytoplasm of several plateau-phase human hematological cell lines (8226, CCRF, H929, HL-60), and that the cytoplasmic translocation paralleled a decrease in sensitivity to etoposide (Valkov et al., 2000; Engel et al., 2004). No difference in the concentration or molecular mass of topo I, IIα or IIβ was observed, indicating that the cytoplasmic location was not a result of protein degradation or of a truncated enzyme. These data may also be clinically relevant, in that topo IIα has been found to have a cytoplasmic distribution in malignant plasma cells from bone marrow aspirates from patients with multiple myeloma (Valkov et al., 2000). In summary, these data suggest that topo IIα may be translocated from the nucleus to the cytoplasm under specific cellular conditions, and this may result in altered drug sensitivity.

Although there is evidence that topo IIα can be transported between the nucleus and cytoplasm (Valkov et al., 2000; Oloumi et al., 2000), the majority of data are limited to describing the import of topo into the nucleus from the cytosol. In fact, there has been only one study that has addressed the mechanism of the nuclear export of topo (Mirski et al., 2003). The results of this study were restricted to predicting the existence of a NES in topo IIα and topo IIβ from peptide studies, and did not define a functional NES in the full-length protein (Mirski et al., 2003). The results of our study demonstrate that human topo IIα contains two functional LMB-sensitive NESs in the full-length protein.

Materials and Methods

Cell culture and accelerated-plateau cell model

HeLa cells were grown in Alpha Minimal Essential Medium (Gibco) containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Hyclone). HL-60 leukemia cells and H929 myeloma cells were grown in RPMI medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Hyclone). The nuclear-cytoplasmic trafficking of topo IIα was examined by plating cells in an accelerated-plateau cell model previously developed in this laboratory (Valkov et al., 2000; Engel et al., 2004). For HL-60 and H929 cells, log-phase conditions are defined by growing cells at 2.0×10^5 cells/ml and plateau-phase cells at 2.0×10^6 cells/ml. Both log and plateau phase cells were grown in fresh medium in a 5% CO_2 incubator at 37°C for 24 hours prior to experiments.

Putative NES peptides

The complete amino acid sequence for human topo IIα (accession number NP 001058) was downloaded from the National Center for Biotechnology Information database and searched for matches to the NES consensus sequence from Table 1. Six amino acid sequences in topo IIα matched the NES consensus sequence (Table 2), and were synthesized as native (nt) or mutated (Δ) peptides. The mutated peptides contain alanine in place of those hydrophobic residues suspected of being critical for nuclear export (leucine, isoleucine, or valine). To facilitate conjugation with preactivated SMCC-BSA, the NES-peptides were designed with a cysteine residue at the amino terminus. The peptides obtained from the Biopeptide Company (San Diego, California) were as follows: ($NES_{80\text{-}91}$), C^{80}GLYKIF-DEILVN^{91}; (mutated $NES_{80\text{-}91}$), $C^{\Delta80}$G<u>A</u>YK<u>A</u>FDE<u>AAA</u>N^{91}; ($NES_{230\text{-}241}$), C^{230}SLDKDIVALMVR^{241}; (mutated $NES_{230\text{-}241}$), $C^{\Delta230}$S<u>A</u>DKD<u>AA</u>A<u>A</u>M<u>A</u>R^{241}; ($NES_{467\text{-}476}$), C^{467}TLAVSGLG-VVG^{477}; (mutated $NES_{467\text{-}477}$), $C^{\Delta467}$T<u>AA</u>SG<u>A</u>G<u>AA</u>G^{477}; ($NES_{1017\text{-}1028}$), C^{1017}DILRDFFELRLK^{1028}; (mutated $NES_{1017\text{-}1028}$), $C^{\Delta1017}$CDI<u>A</u>RD<u>A</u>FE<u>A</u>R<u>A</u>K^{1028}. Peptides ($NES_{569\text{-}580}$), C^{569}FLEE-FITPIVKV^{580}; (mutated $NES_{569\text{-}580}$), $C^{\Delta569}$<u>AA</u>EE<u>AA</u>TP<u>AA</u>K<u>A</u>^{580}; ($NES_{1054\text{-}1066}$), C^{1054}FILEKIDGKIIIE^{1066}; and (mutated $NES_{1054\text{-}1066}$); $C^{\Delta1054}$FI<u>A</u>EK<u>A</u>DGK<u>A</u>I<u>A</u>E^{1066} were obtained from the University of Florida Protein Chemistry Core Facility (Gainesville, FL, USA). All peptides were HPLC purified to >95% and analyzed by mass spectroscopy. In addition, peptide sequences and purity were confirmed by Rick Feldhoff, PhD at the University of Louisville, School of Medicine, Department of Biochemistry (Louisville, KY, USA).

Generation of BSA-peptide-FITC conjugates

Peptides were crosslinked to BSA and FITC as previously described (Stommel et al., 1999). A total of 2 mg Imject® Maleimide activated sulfosuccinmidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate bovine serum albumin (Sulfo-SMCC BSA) (Pierce, Rockford, IL, USA) were reconstituted in 200 μl distilled water. Then, a molar excess (1-2 mg) of native or mutated topo IIα NES peptide in 400 μl of conjugation buffer (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M NaCl, 0.002% sodium azide, pH 7.2) was mixed with the Sulfo-SMCC BSA and reacted for 30 minutes

Table 1. Determination of a concensus sequence from established NES

NES consensus	$HX_{1-4}HX_{2-3}HXH$ (X=Leu, Ile, Val or Phe)											
α-actin (Wada et al., 1998)	A	**L**	P	H	A	**I**	M	R	**L**	D	**L**	A
β-actin (Wada et al., 1998)	A	**L**	P	H	A	**I**	L	R	**L**	D	**L**	A
TFIIIA (Fridell et al., 1996)	–	**L**	–	P	V	**L**	E	N	**L**	T	**L**	–
PKIα (Wen et al., 1995)	E	**L**	A	L	K	**L**	A	G	**L**	D	**I**	N
MAPKK (Fukuda et al., 1996)	A	**L**	Q	K	K	**L**	E	E	**L**	E	**L**	D
RevHIV-1 (Fischer et al., 1995)	Q	**L**	–	P	P	**L**	E	R	**L**	T	**L**	D
Ran BP-1 (Zolotukhin and Felber, 1997)	K	**V**	A	E	K	**L**	E	A	**L**	S	**V**	R
C-Abl (Taagepera et al., 1998)	–	**L**	E	S	N	**L**	R	E	**L**	Q	**I**	C
hZyxin (Nix and Beckerle, 1997)	**L**	T	M	K	E	**V**	E	E	**L**	E	**L**	L
MdM2 (Roth et al., 1998)	S	**L**	S	**F**	D	E	S	–	**L**	A	**L**	C
P53 (Strommel et al., 1999)	**F**	R	E	**L**	N	E	A	L	E	**L**	K	D

Bold letters represent hydrophobic residues thought to be crucial for nuclear export.

Table 2. Putative NES in human topo IIα

(80-90)	–	G	**L**	Y	K	–	**I**	F	D	E	**I**	L	**V**
(230-241)	–	S	**L**	D	K	D	**I**	V	A	**L**	M	**V**	R
(467-476)	–	T	**L**	A	–	–	**V**	S	G	**L**	G	**V**	V
(569-580)	–	F	**L**	E	E	F	**I**	T	P	I	**V**	K	**V**
(1017-1028)	D	I	**L**	R	D	–	**F**	F	E	**L**	R	**L**	K
(1054-1065)	F	I	**L**	E	K	–	**I**	D	G	K	**I**	I	**I**

Bold letters represent hydrophobic residues thought to be crucial for nuclear export.

at room temperature. The reaction was quenched by adding 40 mM cysteine solution in deionized water to the peptide-SMCC-BSA solution to obtain a molar excess of cysteine to peptide sample (approximately 7 nmoles cysteine/nmole of SMCC-BSA-peptide). The conjugates were purified by size exclusion chromatography at room temperature using the Pharmacia P-500 FPLC system with LKB control Unit UV-1. The high resolution column (10 mm inner diameter and 30 cm length) (Amersham Pharmacia, Piscataway, NJ, USA) was packed at 2.0 ml/minute with Superdex 200 prep grade (Amersham Pharmacia) in filtered and degassed PBS, pH 7.4. The peptide conjugates were loaded onto the column using a 500 μl Superloop and were run at 0.5 ml/minute in degassed dH_2O. The 500 μl fractions were collected with a Fraction-100 collector (Pharmacia Biotech) and stored at 4°C overnight. Total protein was estimated in peak samples by measuring the absorbance at 562 nm using the Fisherbrand Protein Assay. Approximately 25 μg of protein from eluted fractions were loaded onto a 10% SDS-PAGE gel and electrophoresed at 7 W for 2-3 hours. Peptide conjugation was confirmed by silver stain analysis. Similar fractions of crosslinked BSA-peptide were pooled and concentrated on a Microsep 30 k filter by centrifuging at 5000 ***g*** in an SS-34 rotor until dry. The samples were eluted with 400 μl of PBS, pH 7.4 to obtain approximately 2 mg/ml peptide-conjugate solution. FITC was solubilized in DMSO at 1 mg/ml and added to the peptide sample in four 5 μl aliquots until a total of 20 μl of FITC (Sigma) were added. FITC was reacted with the peptides for 6 hours at 4°C, and then 23 μl of 1 M NH_4Cl in PBS, pH 7.4 were added to the sample and incubated for 2 hours at 4°C. FITC-BSA-peptide conjugates were separated from unincorporated label by FPLC as described above. The samples were concentrated in a SpeedVac and the ratio of fluorescein to protein was determined by measuring the absorbance at 495 nm and 280 nm. The nuclear control, tetramethylrhodamine-bovine serum albumin (TRITC-BSA), was obtained from Sigma.

Microinjection

To promote cell adherence, Fisherbrand glass coverslips were pretreated with 1 N HCl for a minimum of 4 hours at 50°C and then rinsed extensively with deionized water. Coverslips were washed in 100% ethanol and dried between pieces of Whatman paper. Subconfluent HeLa cells were plated onto the center of glass coverslips in NUNC brand Petri dishes and incubated at 37°C for 24-48 hours preceding microinjection. Prior to microinjection, cells were gently rinsed with sterile PBS warmed to 37°C and replaced with Leibovitz's L-15 medium containing no phenol red.

The peptide samples from above were centrifuged at 13,000 ***g*** for 30 minutes at 4°C, and the supernatant then loaded into Eppendorf Femptotips (diameter of 0.5 μm ± 0.2 μm). All cells were injected using the semi-automated Eppendorf Injectman NI2 and Femtojet microinjector on a Nikon TE 2000 inverted microscope under exactly the same conditions (injection pressure (P_i), 100 hPa; compensation pressure (P_c), 30 hPa; injection time (I_t) 0.2 seconds, atmospheric conditions). Following injection the cells were incubated at 37°C for up to 90 minutes, washed with Leibovitz's L-15 medium, and then fixed with 4% paraformaldehyde for 3 minutes at room temperature, rinsed in PBS and mounted on Shandon microscope slides with mounting medium containing DAPI. Fluorescence was observed with a Leitz Orthoplan 2 microscope and images were captured using a CCD camera with Smart Capture program (Vysis, Downers Grove, IL, USA).

Topoisomerase IIα cloning and site-directed mutagenesis

Topo IIα primers were designed that contained the eight amino acid FLAG peptide preceded by a start codon and a Kozak motif (5′-ATG GAC TAC AAA GAC GAT GAC GAC AAG GAA GTG TCA CCA TTG CAG CCT GTA AAT GAA AAT ATG-3′ forward primer, 5′-ATG CGG CCG CTT AAA ACA GAT CAT CTT CAT CTG ACT CTT C-3′ reverse primer). Amplification was performed with an enzyme mixture of *Taq* and *Pyrococcus* species GB-D thermostable DNA polymerases (Elongase, Invitrogen), in 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM dNTP mixture and 200 nM each primer. Forty cycles were performed (94°C for 30 seconds, 60°C for 30 seconds and 5.5 minutes at 68°C) and the PCR products were agarose gel-purified and ligated to a pcDNA3.1 vector (CMV promoter) using a TOPO-TA cloning system (Invitrogen). The 5′ end of the new FLAG-topo IIα fusion protein vector was sequenced to ensure that the DNA was in-frame. Site-directed mutagenesis was

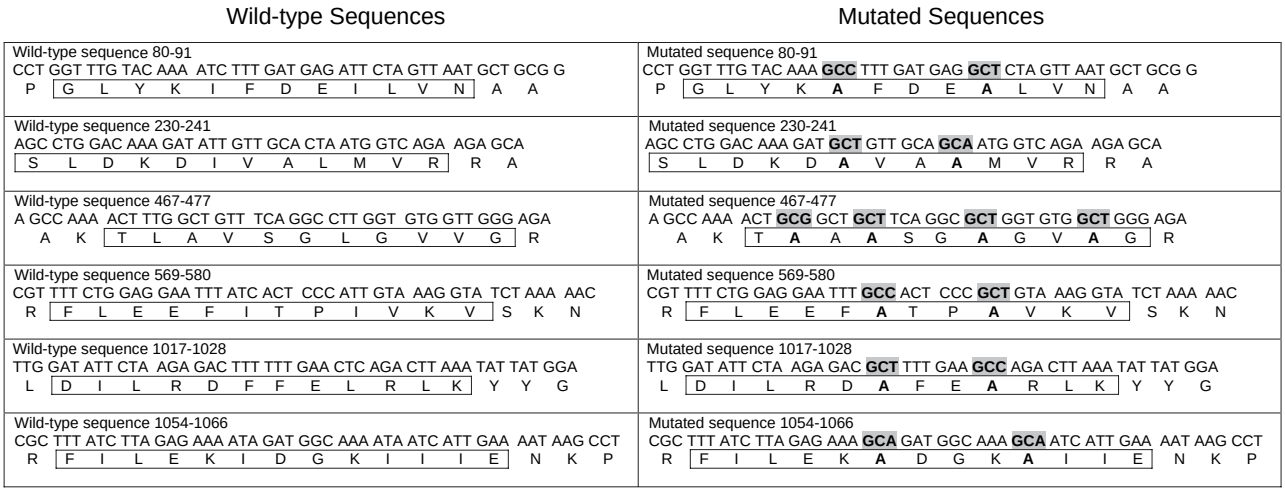

Fig. 1 Site-directed mutagenesis. The figure compares nucleotide sequences from the wild-type topo IIα gene and the mutated sequences used in this study. Hydrophobic amino acid residues thought to be necessary for nuclear export were mutated to alanine using site-directed mutagenesis (gray boxes). Putative NES amino acid sequences are in boxes below their corresponding nucleotide sequences.

performed using a Quickchange XL site-directed mutagenesis kit (Stratagene). Briefly, 100 ng of template dsDNA were mixed with 125 ng of each oligonucleotide primer, 2.5 units of *PfuTurbo* DNA polymerase, in a reaction mixture containing 2 mM dNTPs. Eighteen cycles were performed (95°C for 50 seconds, 60°C for 50 seconds and 68°C for 20 minutes), after which the parental plasmid was digested with methylation-specific enzyme *Dpn-I*. Ultra-competent cells were transformed with mutated plasmid and clones were sequenced to determine the presence of desired mutations. DNA sequencing was performed at the H. Lee Moffitt Cancer Center Molecular Biology Core Facility. Primers containing mutant sequences are listed in Fig. 1.

Transfection protocol

Human myeloma H929 and HL-60 cells (ATCC) were plated at log phase density (2×10^5 cells/ml) 2 days prior to transfection. Transfection was performed as previously described (Van den Hoff et al., 1992). Briefly, 40 μg of wild-type or mutated topo IIα plasmid in 300 μl of a solution containing 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, were precipitated by the addition of 30 μl of 5 M NaCl and two volumes of 95% ethanol on ice for 10 minutes. Plasmid was pelleted by centrifugation for 15 minutes at 20,000 ***g*** at 4°C, washed with 75% ethanol and re-centrifuged. All remaining ethanol was removed using a pipette and the DNA immediately resuspended in 50 μl of cytomix buffer containing 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP and 5 mM glutathione, and the pH was adjusted to 7.6 by the addition of KOH. ATP and glutathione were made fresh and added prior to each transfection (Van den Hoff et al., 1992). Two days prior to transfection, human myeloma H929 cells and leukemia HL-60 cells were placed in fresh growth medium (RPMI/10% FBS/pen-strep) at a concentration of 2×10^5 cells/ml. Cells were collected and 1.6×10^7 cells pelleted by centrifugation at 1500 ***g*** for 5 minutes. Cell pellets were washed twice in 10 ml of sterile PBS, resuspended in 350 μl of cytomix buffer (4°C), mixed with prepared DNA and placed in a 4 mm electroporation cuvette. Electroporation was at 250V/750 capacitance, after which cells were split into even groups and plated at log (2×10^5 cells/ml) and plateau (2×10^6 cells/ml) growth conditions for 20 hours in a 5% CO_2 incubator at 37°C with RPMI medium containing 5% FBS.

Immunofluorescence

Twenty hours post-transfection, viable H929 and HL-60 cells were isolated by centrifugation at 2000 ***g*** for 20 minutes at 20°C on a Ficoll gradient and washed with PBS. Transfected cells were plated on a glass microscope slide using cytospin funnels and fixed with 4% paraformaldehyde at 20°C for ten minutes. The fixation was stopped by washing in PBS and cells were permeabilized for 24 hours in a solution containing 1% glycine and 0.25% Triton X-100 in PBS. Slides were stained with anti-FLAG M2 monoclonal antibody-FITC conjugate (Sigma) diluted 1:100 with 0.1% NP-40 and 1% BSA in PBS, and incubated for 1 hour at room temperature. Slides were washed in PBS, dried briefly and counterstained with Vectashield mounting medium antifade/DAPI (1:1) (Vector Laboratories Inc., Burlingame, CA, USA). Immunofluorescence was observed with a Leitz Orthoplan 2 fluorescent microscope and images were captured by a CCD camera with Smart Capture program (Vysis, Downers Grove, IL). Quantitation of FITC fluorescence was performed using the Adobe Photoshop 7.0 program.

Western blot analysis

HeLa cells grown in RPMI medium containing 5% FBS were transfected directly on 100 cm^2 tissue culture plates. Plasmid DNA (10 μg) was mixed with 60 μl of Superfect transfection reagent (Qiagen) in 300 μl of serum-free medium for 10 minutes, followed by 600 μl of serum containing medium, and the entire mixture was then added directly to cell culture plates. Transfection was allowed to proceed for 3 hours at 37°C in a 5% CO_2 incubator and terminated by the removal of transfection solution and the addition of 15 ml of 5% FBS-containing RPMI medium. After incubation for 24 hours, the cells were harvested by the addition of 0.53 mM EDTA, washed with cold PBS, and lysed in SDS buffer (2% SDS, 10% glycerol, 0.06 M Tris, pH 6.8). Protein from 2×10^5 cells per lane was separated on an 8% SDS-PAGE gels and electroblotted (Biorad) onto nitrocellulose membranes (Amersham). The blots were blocked for 1 hour at ambient temperature in a blocking buffer containing 0.1 M Tris-HCl-buffered saline, 0.5% Tween 20, and 5% non-fat milk. Blots were stained by the direct addition of anti-FLAG M2 (Sigma) antibody and incubated overnight at 4°C. Membranes were washed three times for 10 minutes with 0.1 M Tris-HCl-buffered saline and incubated with anti-mouse IgG

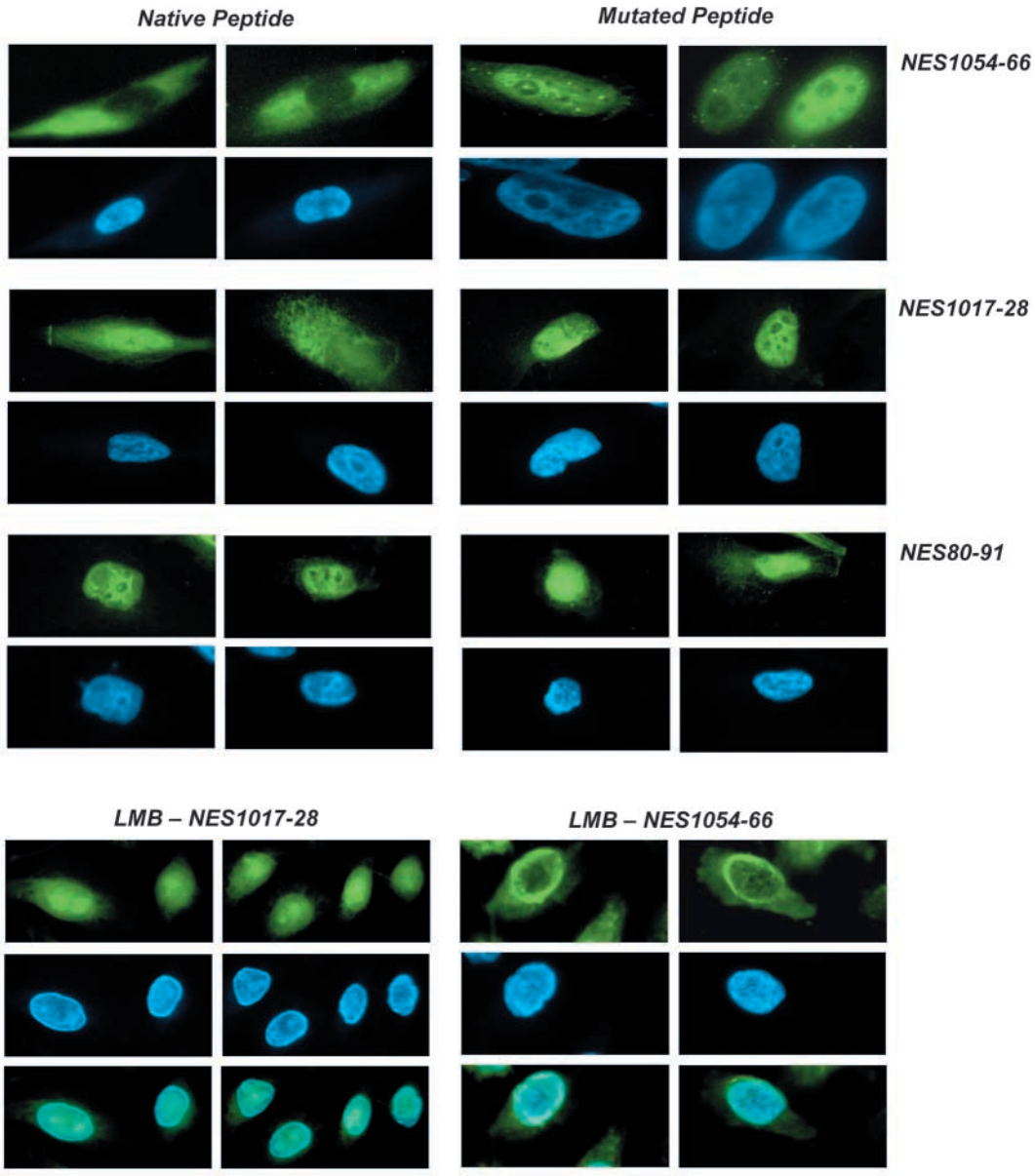

Fig. 2. HeLa cells microinjected with either wild-type (left column) or mutated (right column) peptide-BSA-FITC conjugates (green), and then counterstained with DAPI (blue). A total of 20-50 cells were successfully microinjected per peptide and similar results were seen in all cells. The bottom of the figure presents HeLa cells that were microinjected with wild-type peptide-BSA-FITC conjugates in the presence of 2 ng/ml LMB (leptomycin B). Panels showing mutated peptide BSA-NES 1054-66 are at a higher magnification than the other micrographs.

antibody (Sigma) in 0.1 M Tris-HCl buffered saline, 0.5% Tween 20 and 5% non-fat milk for 60 minutes at room temperature. Antibody binding was visualized by ECL (Amersham) on autoradiography film (Kodak).

Results

Peptides $NES_{1054-1066}$ and $NES_{1017-1028}$ signal the nuclear export of BSA-FITC

Of the six putative NES identified in topo IIα, two peptides, $NES_{1054-1066}$ and $NES_{1017-1028}$, signaled the export of BSA into the cytoplasm when microinjected into the nuclei of HeLa cells (Fig. 2). BSA-$NES_{1054-1066}$ showed strong cytoplasmic staining and was seen in the cytoplasm within 15 minutes of microinjection, as compared to TRITC-BSA alone (not shown), or the mutated BSA-$NES_{1054-1066}$ conjugate. BSA-$NES_{1017-1028}$ also appeared cytoplasmic within 15 minutes of being microinjected into the nucleus, but complete nuclear clearing (seen with BSA-$NES_{1054-1066}$) was not observed even after 90 minutes. The mutated BSA-$NES_{1017-1028}$ was nuclear in all cells even 90 minutes after microinjection. BSA-NES_{80-90} (Fig. 2), mutated BSA-NES_{80-90} (Fig. 2), BSA-$NES_{230-241}$, mutated BSA-$NES_{230-241}$, and BSA-$NES_{467-476}$, BSA-$NES_{569-580}$, and mutated BSA-$NES_{569-580}$ all remained in the nucleus even 4 h after microinjection (data not shown).

LMB blocks $NES_{1054-1066}$ and $NES_{1017-1028}$ mediated nuclear export

Leptomycin B (LMB) (Hamamoto et al., 1983a; Hamamoto et al., 1983b; Hamamoto et al., 1985) is a specific inhibitor of CRM-1-mediated nuclear export of proteins (Nishi et al., 1994). To determine if the nuclear export of BSA conjugated to peptides $NES_{1054-1066}$ and $NES_{1017-1028}$ was CRM-1 dependent, LMB-pretreated HeLa cells were microinjected in the presence of 2 ng/ml LMB in ethanol. Fluorescence

microscopy demonstrated that LMB blocked the export of BSA conjugated to peptides $NES_{1054-1066}$ and $NES_{1017-1028}$ (Fig. 2). $BSA\text{-}NES_{1054-1066}$ had a strong perinuclear staining, suggesting that the protein cargo was docking at the NPC (Siomi et al., 1997; Arlucea et al., 1998).

Although the NES defined by microinjection are sufficient to transport a non-shuttled protein to the cytoplasm, these leucine rich sequences may or may not serve a role in exporting topo IIα. To determine if these NES are necessary for topo IIα export, we observed the trafficking of FLAG-topo IIα expressed in human myeloma cell lines in the accelerated-plateau cell system.

Topoisomerase IIα cloning, site directed mutagenesis, and gene expression

We first attempted to study full-length topo IIα protein trafficking using a green fluorescent protein-topoisomerase II alpha (GFP-topo IIα) fusion protein expression vector. However, we found that expression of GFP-topo IIα recombinant protein was cytotoxic, inducing apoptosis in all cell lines tested (HeLa, HL-60, H992, 8226, MCF-7, Chinese hamster ovary) 16-48 hours after transfection. In addition, translocation of GFP-topo IIα to the cytoplasm in plateau density cells was extremely limited when compared to endogenous topo IIα (data not shown). GFP-topo IIα plasmid containing site-directed mutations of the six putative nuclear export signals listed in Fig. 1, showed minimal change in export when compared to wild-type GFP-topo IIα (data not shown). Additionally, we produced a GFP-topo IIα fusion plasmid in which the active-site, tyrosine 805, was mutated to an alanine. GFP-topo IIα A805 gave similar negative results; it was lethal to the transfected cells and minimally exported to the cytoplasm in plateau density cells (contrary to endogenous topo IIα). We made one additional plasmid with a destabilized GFP-topo IIα fusion protein, theorizing that the lethality of the fusion protein may be due to accumulation of GFP-topo IIα in the transfected cells. A destabilized GFP fusion protein is turned over every 4 hours in transfected cells, thus not allowing cellular accumulation of recombinant protein to cytolytic levels. Destabilized GFP-topo IIα protein was also cytotoxic and did not translocate to the cytoplasm in plateau density cells.

The lack of success with GFP led us to look for alternative topo IIα fusion proteins, such as the FLAG peptide. FLAG peptide is an eight amino acid protein (NYKNNNNK) that does not occur in nature. FLAG does not contain any putative nuclear export signals and its small size limits any secondary protein structure problems. HeLa cells transfected with FLAG-topo IIα plasmid vectors express full-length (170 kDa) topo IIα recombinant proteins (Fig. 3).

Since our primary interest was human myeloma cells, we encountered another technical problem inherent with these cells, which is difficulty in transfecting them. In an attempt to transfect human myeloma cell lines, we tried a number of commercially available transfection reagents and numerous protocols, with no success. The only technique that yielded positive transfectants was a method described by Van den Hoff et al. (Van den Hoff et al., 1992). This method utilizes a cytomix buffer made to approximate the intracellular environment (see Materials and Methods). In addition, this buffer contains ATP and glutathione to promote the rapid repair of cellular membranes. We were able to transfect cells with a high degree of efficiency for these cell lines (2-20%) using this method.

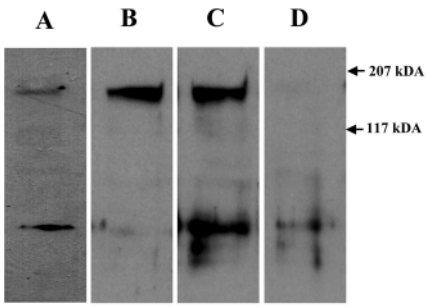

Fig. 3. Western blot of full-length FLAG-topo IIα protein. HeLa cells were transfected with plasmid containing FLAG-topo IIα plasmid via cationic lipid, and harvested after 20 hours. Protein extracts from 2×10^5 cells per lane were separated on a SDS-PAGE gel, blotted onto nitrocellulose and probed with FLAG M2 antibody. Lane A is protein from cells transfected with non-mutated FLAG-topo IIα plasmid, lane B is FLAG-topo IIα plasmid 1054-1066, lane C transfected with FLAG-topo IIα plasmid 1017-1028, and lane D is a non-transfected control.

FLAG-topoisomerase IIα immunofluorescence

BSA-peptide microinjection data indicated that the putative nuclear export sites at 1017-1028 and 1054-1066 may function to signal export of topo IIα. To confirm these data with a full-length topo IIα protein, H929 human myeloma cells were transfected with FLAG-topo IIα expression vectors possessing mutated hydrophobic residues in the nuclear export sites at 1017-1028 and 1054-1066 (Fig. 1). Twenty hours post-transfection, viable cells were isolated by centrifugation on a Ficoll-paque gradient and plated on glass microscope slides using cytospin funnels. After fixing and permeabilization, slides were stained with anti-FLAG M2 monoclonal antibody-FITC conjugate and counterstained with mounting medium containing DAPI to show the location of the nuclei. Images were acquired using a fluorescence microscope (Fig. 4), with quantitation of FITC fluorescence using Adobe Photoshop 7.0 (data presented in Fig. 5). Fig. 4 establishes that the wild-type (non-mutated) FLAG-topo IIα protein is present in the nucleus of the cells plated at log density, whereas FLAG-topo IIα protein is located in the cytoplasm in cells plated at plateau density. Quantitation of fluorescence revealed a statistically significant shift (P=0.00001) for log cells with a nuclear:cytoplasmic ratio of 5.9:1, to a ratio of 0.42:1 in plateau cells (Fig. 5) when using the wild-type FLAG-topo IIα plasmid. When the putative export signals at either 1017-1028 (Fig. 4) or 1054-1066 were mutated, export to the cytoplasm was abrogated. Quantitative analysis of fluorescence of both mutant proteins (1017-1028 or 1054-1066) revealed no statistically significant change in the levels of nuclear or cytoplasmic FLAG-topo IIα in log or plateau density cell cultures. Even though export to the cytoplasm of mutant 1054-1066 was abrogated, qualitatively it appeared that the mutated FLAG-topo IIα protein was localized predominantly at the nuclear membrane as compared to mutant 1017-1028. The putative signal at 467-476 was similar to wild-type (Fig. 4) as were putative signals at amino acid 80-91, 230-241 and 569-580 (data not shown).

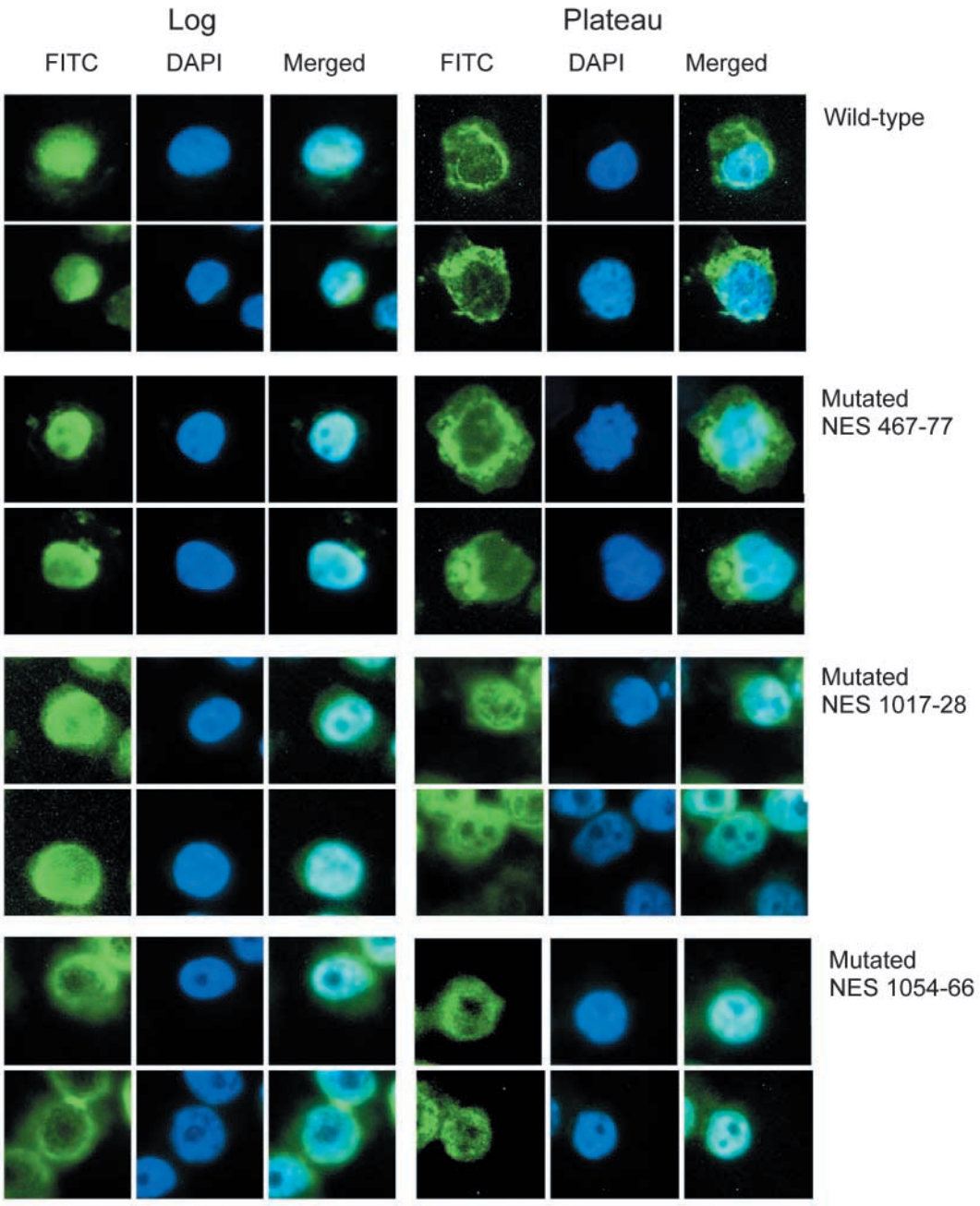

Fig. 4. FLAG-topo IIα immunofluorescence. Human multiple myeloma H929 cells were transfected by electroporation with full-length wild-type and mutated topo IIα and plated for 20 hours at log and plateau cell densities. Cytospins containing fixed cells were stained with FITC-labeled anti-FLAG M2 antibody (green), counterstained with DAPI for nuclear staining, and assayed by immunofluorescent microscopy.

Peptide $NES_{1054\text{-}1066}$ and $NES_{1017\text{-}1028}$ are conserved

To determine if peptides $NES_{1054\text{-}1066}$ and $NES_{1017\text{-}1028}$ are conserved, a BLAST search of the SWISS-PROT database was performed to identify homologous sequences in topo IIα. Tables 3 and 4 summarize a list of representative species containing homologous topo IIα sequences. The data show that the characteristic spacing of hydrophobic residues in peptides $NES_{1054\text{-}1066}$ and $NES_{1017\text{-}1028}$ are highly conserved in a broad range of species. For example, leucine residues appearing in human topo IIα NES are often substituted with the hydrophobic amino acids isoleucine or valine. Furthermore, Phe^{1054} and Ile^{1055} in peptide $NES_{1054\text{-}1066}$ are highly conserved from mammals to the most primitive eukaryotic organism, *Giardia lamblia*, unlike Leu^{1056}. This suggests that the presence of phenylalanine and isoleucine are critical for nuclear export of this peptide, and thus an omission of these two hydrophobic amino acids from the peptide sequence could explain why a previous report failed to identify $NES_{1054\text{-}1066}$ as a nuclear export signal (Mirski et al., 2003).

$NES_{1054\text{-}1066}$ and $NES_{1017\text{-}1028}$ reside within a putative coiled-coil domain

We were interested in predicting the structural features of the region containing $NES_{1017\text{-}1028}$ and $NES_{1054\text{-}1066}$. Each topo II monomer can be divided into three domains, an N-terminal domain that contains the ATP-binding region, the central domain containing the active site tyrosine residue, and the C-terminal domain that contains the nuclear localization sequences (Watt and Hickson, 1994). Both of the NES are

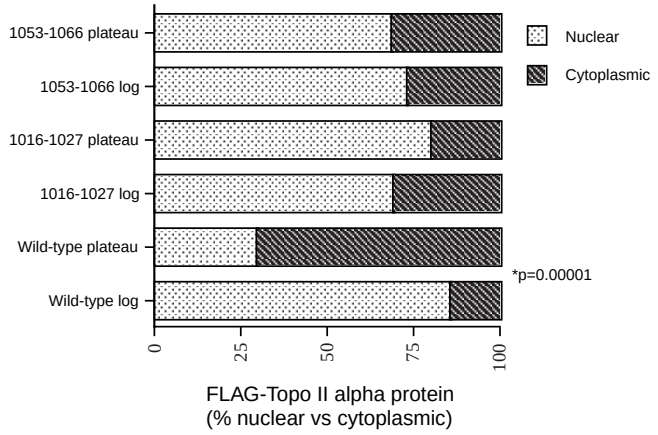

Fig. 5. Nuclear export of wild-type and mutant FLAG-topo IIα plasmids. Human myeloma H929 transfected cells (*n*=20) stained with anti-FLAG M2 monoclonal antibody-FITC conjugate were assayed for nuclear and cytoplasmic immunofluorescence. Quantitation of FITC fluorescence was performed using Adobe Photoshop 7.0 program. Wild-type FLAG-topo IIα was exported to the cytoplasm in cells at plateau density (*P*=0.00001), whereas topo IIα mutated at the putative export sites, 1016-1027 and 1053-1066, did not demonstrate statistically significant levels of export to the cytoplasm.

situated upstream of the bipartite NLS (Fig. 6) and downstream of the active-site tyrosine residue (Tyr^{805}). Furthermore, several CK-2 phosphorylation sites downstream of both NES have been identified in vitro and could be important for regulating the subcellular localization of topo IIα. Although $NES_{1017-1028}$ alone is predicted to form an α-helix (Mirski et al., 2003), we were interested in predicting the motif of the complete amino acid sequence stretching from $NES_{1017-1028}$ to $NES_{1054-1066}$. According to EMBOSS and Predict Protein, two programs designed to predict protein motifs, amino acids 1017-1066 are characterized by a high potential to form α-helices and also contain five 4-3 hydrophobic repeats, a typical feature of a coiled-coil motif. Such a repeating pattern of hydrophobic amino acids has been shown to form a hydrophobic core, which is critical for dimerization (Sodek et al., 1972). In this manner, the hydrophobic amino acids are predicted to align on the same interface that facilitates DNA binding or protein-protein interactions. Interestingly, amino acids 1013-1056 in topo IIα have previously been shown to form a stable two-stranded α-helical coiled-coil in solution (Frere et al., 1995; Frere-Gallois et al., 1997; Bjergbeck et al., 1999). Perhaps more importantly than the predictive data above, the crystal structure of topo II from *Saccharomyces cervisiae* was shown to have primary and secondary dimerization domains (Fass et al., 1999). The primary dimerization region is highly conserved and corresponds to amino acids 1013-1056 in human topo IIα (Frere et al., 1995).

Discussion

Human topo I (Mo et al., 2000), IIα and IIβ (Mirski et al., 1999) have NLSs that target their movement into the nucleus, but only one report has begun to address the mechanism of nuclear export of topo IIα and topo IIβ (Mirski et al., 2003). The nuclear export of topo I in response to topotecan or camptothecin exposure has been reported (Danks et al., 1996), as has the redistribution of topo I from the nucleus to the nucleoli (Buckwalter et al., 1996). The lack of data describing the nuclear-cytoplasmic shuttling of topo enzymes may be because topo is usually found to occur in the nucleus of cells, and a cytoplasmic distribution of topo IIα has only been attributed to the expression of a truncated protein that has lost its C-terminal NLS. However, proteins that appear predominately nuclear may still shuttle between the nucleus and the cytoplasm, if the rate of nuclear import is greater than the rate of nuclear export. Thus, demonstrating that a protein shuttles between the nucleus and cytoplasm requires defining the specific conditions that will shift the steady state kinetics toward nuclear export. Many conditions have been shown to alter the shuttling of proteins between the nucleus and

Table 3. Sequence alignment of topo IIα $NES_{1017-1028}$

Homo sapiens (human) $NES_{1017-1028}$	D	**I**	**L**	R	D	**F**	F	E	**L**	R	**L**	K
Sus scrofa (pig), *Mus musculus* (mouse), *Rattus norvegicus* (rat)	D	**I**	**L**	R	D	**F**	F	E	**L**	R	**L**	K
Cricetulus griseus (Chinese hamster)	D	**I**	**L**	–	D	**F**	F	E	**L**	R	**L**	K
Gallus gallus (chicken)	D	**I**	**L**	–	–	**F**	F	E	**L**	R	**L**	–
Saccharomyces cerevisiae (yeast)	–	**I**	**L**	–	–	**F**	–	–	**V**	R	**L**	–
Aspergillus niger (fungus), *penicillium citrinum* (fungus)	D	**I**	**L**	–	–	**F**	F	–	**V**	R	**L**	K
Nicotiana tabacum (tobacco)	D	**I**	**L**	–	–	**F**	–	–	**V**	R	**L**	–
Encephalitozoon cuniculi (protozoan)	–	**I**	**L**	–	–	**F**	–	–	**V**	R	**L**	–
Bombyx mori (silkmoth)	–	**I**	**L**	R	–	**F**	–	–	**L**	R	**V**	–

Bold letters represent hydrophobic residues thought to be crucial for nuclear export.

Table 4. Sequence alignment of DNA topo IIα $NES_{1054-1066}$

Homo sapiens (human) $NES_{1054-1066}$	**F**	**I**	L	E	K	**I**	D	G	K	**I**	I	**I**	E
Cricetulus griseus (Chinese hamster), *Sus scrofa* (pig)	**F**	**I**	L	E	K	**I**	D	G	K	**I**	I	**I**	E
Gallus gallus (chicken), *Mus musculus* (mouse), *Rattus norvegicus* (rat)	**F**	**I**	L	E	K	**I**	D	G	K	**I**	V	**I**	E
Caenorhabditis elegans (nematode)	**F**	**I**	L	–	K	**I**	–	–	–	**I**	V	**L**	E
Saccharomyces cerevisiae (yeast)	**F**	**I**	–	–	–	**I**	–	–	–	**L**	–	**V**	–
Candida glabrata (yeast)	**F**	**I**	–	–	–	**I**	–	–	–	**L**	I	**V**	–
Aspergillus candidus (fungus), *Trichophyton rubrum* (fungus)	**F**	**V**	–	–	–	**I**	–	G	–	**L**	V	**V**	–
Giardia lamblia (protist)	**F**	**I**	–	–	–	**I**	–	–	–	**L**	–	**I**	–

Bold letters represent hydrophobic residues thought to be crucial for nuclear export.

Fig. 6. The complete amino acid sequence of human DNA topo IIα (accession number NP 001058). The active-site tyrosine residue (805) is indicated (*); $NES_{1017\text{-}1028}$, C^{1017}DILRDFFELRLK^{1028} and $NES_{1054\text{-}1066}$, C^{1054}FILEKIDGKIIIE^{1066} are shaded; bipartite NLS is single underlined; Ser/Thr CK2 phosphorylation sites are italicized; the predicted coiled-coil region is boxed.

cytoplasm, including changes in the cell cycle and oxidative stress (reviewed by Damelin et al., 2002). For example, in the accelerated-plateau cell model (Valkov et al., 2000; Engel et al., 2004) used in the present experiments, it is likely that intensive cell-cell contact initiates a signal that induces the export of topo IIα to the cytoplasm (Nix and Beckerle, 1997; Gottardi et al., 1996). This is supported by the findings of others that a cytoplasmic distribution of topo IIα occurs in the outer-proliferating cells of multi-cell spheroids in xenograft tumors when compared to monolayers formed by these cells (Oloumi et al., 2000). Phosphorylation has also been shown to be important in regulating the subcellular localization of many proteins and could have a role in topo IIα trafficking. This is suggested by the finding that the cells of multi-cell spheroids contain a cytoplasmic pool of topo IIα and have a tenfold decrease in the phosphorylation state of the enzyme when compared to monolayers (Oloumi et al., 2000). Since CK-2 has been shown to phosphorylate topo IIα on several serine and threonine residues near the NES or NLS (Ackerman et al., 1985), CK-2 is a logical candidate for modulating topo IIα trafficking.

One of the potential consequences of exporting a pool of topo IIα to the cytoplasm is a decrease in sensitivity to topo poisons. This could result from cytoplasmic topo IIα serving as a drug sink, by trapping VP-16 in this compartment (Ernst et al., 2000). This is supported by data demonstrating that the binding of VP-16 to topo II can occur in the absence of DNA (Burden et al., 1996). For this to occur, the amount of drug binding to topo IIα would need to be sufficient to result in a decrease in drug-induced DNA damage (Burden et al., 1996). Another possibility is that the shuttling of topo IIα to the cytoplasm results in a decrease in the amount of nuclear enzyme available to form enzyme-drug-DNA ternary complexes. In either case, blocking the export of topo IIα with drugs such as LMB may sensitize cells to topo poisons by maintaining the amount of drug target in the nucleus. Although LMB has been shown to have undesirable cytotoxic effect in clinical trials, synthetic derivatives of LMB have become available and may be promising alternatives to LMB therapy (Kalesse et al., 2001; Koster et al., 2003) in hematological malignancies.

Resistance to chemotherapeutic drugs is a major obstacle in the treatment of leukemia and myeloma. We have previously reported that resistance to topoisomerase IIα poisons such as VP-16 was found to increase dramatically with concurrent increases in cell density (Valkov et al., 2000; Engel et al., 2004). Myeloma and leukemic cells in transition from low-density log phase conditions to high-density plateau phase conditions exhibited a substantial export of endogenous topoisomerase IIα from the nucleus to the cytoplasm. In order for topoisomerase-targeted chemotherapy to function, the topoisomerase target must have access to the nuclear DNA. Thus, the nuclear export of topoisomerase IIα must be added to the list of potential mechanisms of resistance to topo poisons. It is unique in that it does not require drug exposure and may mimic the high cell density microenvironment seen in the bone marrow of patients with multiple myeloma. Further defining this mechanism, and possibly modulating export, may lead to methods to preclude this avenue of resistance.

Supported in part by NIH grant CA82533. We would like to thank the Moffitt Cancer Center Analytic Microscopy and Molecular Biology Cores for their expertise and assistance. In addition we would like to express our gratitude to Dr Scott Kaufman and Dr Richard Pollenz for their valuable advice.

References

Ackerman, P., Glover, C. V. and Osheroff, N. (1985). Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity in vitro. *Proc. Natl. Acad. Sci. USA* **82**, 3164-3168.

Arlucea, J., Andrade, R., Alonso, R. and Aréchaga, J. (1998). The nuclear basket of the nuclear pore complex is part of a higher-order filamentous network that is related to chromatin. *J. Struct. Biol.* **124**, 51-58.

Bairoch, A. and Apweiler, R. (2000). The SWISS-PROT protein sequence database and its supplement TREMBL in 2000. *Nucleic Acids Res.* **28**, 45-48.

Bjergbeck, L., Jensen, S., Westergaard, O. and Andersen, A. (1999). Using a biochemical approach to identify the primary dimerization regions in human DNA topoisomerase IIα. *J. Biol. Chem.* **274**, 26529-26536.

Buckwalter, C. A., Lin, A. H., Tanizawa, A., Pommier, Y. G., Cheng, Y. and Kaufmann, S. H. (1996). RNA synthesis inhibitors alter the subnuclear distribution of DNA topoisomerase I. *Cancer Res.* **56**, 1674-1681.

Burden, D. A., Kingma, P. S., Froelich-Ammon, S. J., Bjornsti, M. A., Patchan, M. W., Thompson, R. B. and Osheroff, N. (1996). Topoisomerase II: etoposide interactions direct the formation of drug-induced enzyme-DNA cleavage complexes. *J. Biol. Chem.* **271**, 29238-29244.

Cokol, M., Nair, R. and Rost, B. (2000). Finding nuclear localization signals. *EMBO Rep.* **1**, 411-415.

Cronshaw, J. M., Krutchinsky, A. N., Zhang, W., Chait, B. T. and Matunis, M. J. (2002). Proteomic analysis of the mammalian nuclear pore complex. *J. Cell Biol.* **5**, 915-927.

Cowell, I. G., Willmore, E., Chalton, D., Marsh, K. L., Jazrawi, E., Fisher, L. M. and Austin, C. A. (1998). Nuclear distribution of human DNA topoisomerase IIβ: a nuclear targeting signal resides in the 116-residue C-terminal tail. *Exp. Cell Res.* **243**, 232-240.

Damelin, M., Silver, P. A. and Corbett, A. H. (2002). Nuclear protein transport. *Methods Enzymol.* **351**, 587-607.

Danks, M. K., Garrett, K. E., Marion, R. C. and Whipple, D. O. (1996). Subcellular redistribution of DNA topoisomerase I in anaplastic astrocytoma cells treated with topotecan. *Cancer Res.* **56**, 1664-1673.

Dreger, M. (2003). Subcellular proteomics. *Mass Spectrom.* **22**, 27-56.

Engel, R., Valkov, N. I. and Sullivan, D. M. (2003). In vitro and in vivo drug resistance to DNA topoisomerase I and II inhibitors. *Recent Research Developments in Cellular Biochemistry* **1**, 207-232.

Engel, R., Valkov, N. I., Gump, J. L., Hazlehurst, L., Dalton, W. S. and Sullivan, D. M. (2004). The cytoplasmic trafficking of DNA topoisomerase IIα correlates with etoposide resistance in human myeloma cells. *Exp. Cell Res.* **295**, 421-431.

Ernst, A. I., Soltermann, A., Sigrist, J. A., Widmer, L., Gasser, S. M. and Stahel, R. A. (2000). Ectopic expression of human topoisomerase IIα fragments and etoposide resistance in mammalian cells. *Int. J. Cancer* **88**, 99-107.

Fass, D., Bogden, C. E. and Berger, J. M. (1999). Quaternary changes in topoisomerase II may direct orthogonal movements of two DNA strand. *Nat. Struct. Biol.* **6**, 322-326.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 463-473.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Frere, V., Sourgen, F., Monnot, M., Troalen, F. and Fermandjian, S. (1995). A peptide fragment of human DNA topoisomerase II alpha forms a stable coiled-coil structure in solution. *J. Biol. Chem.* **270**, 17502-17507.

Frere-Gallois, V., Krebs, D., Scala, D., Troalen, F. and Fermandjian, S. (1997). Peptide fragments of DNA topoisomerase II with helix-forming and coiled-coil-forming properties act as inhibitors of the enzyme forms a stable coiled-coil structure in solution. *Eur. J. Biochem.* **249**, 142-148.

Fridell, R. A., Fisher, U., Luhrmann, R., Meyer, B. E., Meinkoth, J. L., Malim, M. H. and Cullen, B. R. (1996). Amphibian transcription factor IIIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 rev. *Proc. Natl. Acad. Sci. USA* **93**, 2936-2940.

Fukuda, M., Gotoh, I., Gotoh, Y. and Nishida, E. (1996). Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.* **271**, 20024-20028.

Fukuda, M., Asano S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311.

Görlich, D. (1998). Transport into and out of the cell nucleus. *EMBO J.* **17**, 2721-2727.

Gottardi, C. J., Arpin, M., Fanning, A. S. and Louvard, D. (1996). The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. *Proc. Natl. Acad. Sci. USA* **93**, 10779-10784.

Hamamoto, T., Gunji, S., Tsuji, H. and Beppu, T. (1983a). Leptomycins A and B, new antifungal antibiotics. I. Taxonomy of the producing strain and their fermentation, purification and characterization. *J. Antibiot.* **36**, 639-645.

Hamamoto, T., Seto, H. and Beppu, T. (1983b). Leptomycins A and B, new antifungal antibiotics. II. Structure elucidation. *J. Antibiot.* **36**, 646-650.

Hamamoto, T., Uozumi, T. and Beppu, T. (1985). Leptomycins A and B, new antifungal antibiotics. III. Mode of action of leptomycin B on Schizosaccharomyces pombe. *J. Antibiot.* **38**, 1573-1580.

Harker, W. G., Slade, D. L., Parr, R. L. and Holguin, M. H. (1995). Selective use of an alternative stop codon and polyadenylation signal within intron sequences leads to a truncated topoisomerase II alpha messenger RNA and protein in human HL-60 leukemia cells selected for resistance to mitoxantrone. *Cancer Res.* **55**, 4962-4971.

Hazelhurst, L. A., Valkov, N. I., Wisner, L., Storey, J. A., Boulware, D., Sullivan, D. M. and Dalton, W. S. (2001). Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. *Blood* **98**, 1897-1903.

Kalesse, M., Christmann, M., Bhatt, U., Quitschalle, M., Claus, E., Saeed, A., Burzlaff, A., Kasper, C., Haustedt, L. O., Hofer, E. et al. (2001). The chemistry and biology of ratjadone. *Chembiochem.* **9**, 709-714.

Kang, M. R. and Chung, I. K. (2002). Down-regulation of DNA topoisomerase II alpha in human colorectal carcinoma cells resistant to a protoberberine alkaloid, berberrubine. *Mol. Pharmacol.* **4**, 879-884.

Kau, T. R. and Silver, P. A. (2003). Nuclear transport as a target for cell growth. *Drug Discov. Today* **8**, 78-85.

Koster, M., Lykke-Andersen, S., Elnakady, Y. A., Gerth, K., Washausen, P., Hofle, G., Sasse, F., Kjems, J. and Hauser, H. (2003). Ratjadones inhibit nuclear export by blocking CRM1/exportin 1. *Exp. Cell Res.* **286**, 321-331.

La Cour, T., Gupta, R., Ropacki, K., Skriver, K., Poulsen, F. M. and Burnak, S. (2003). NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res.* **31**, 393-396.

Low, R. L., Orton, S. and Friedman, D. B. (2003). A truncated form of DNA topoisomerase IIβ associates with the mtDNA genome in mammalian mitochondria. *Eur. J. Biochem.* **270**, 4173-4186.

Mirski, S. E., Gerlach, J. H. and Cole, S. P. (1999). Sequence determinants of nuclear localization in the alpha and beta isoforms of human topoisomerase II. *Exp. Cell Res.* **251**, 329-339.

Mirski, S. E., Bielawski, J. C. and Cole, S. P. (2003). Identification of functional nuclear export sequences in human topoisomerase II alpha and beta. *Biochem. Biophys. Res. Commun.* **306**, 905-911.

Mo, Y-Y., Wang, C. and Beck, W. T. (2000). A novel nuclear localization signal in human DNA topoisomerase I. *J. Biol. Chem.* **275**, 41107-41113.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S. and Beppu, T. (1994). Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J. Biol. Chem.* **269**, 6320-6324.

Nix, D. A. and Beckerle, M. C. (1997). Nuclear-cytoplasmic shuttling of the focal contact protein, zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus. *J. Cell Biol.* **138**, 1139-1147.

Oloumi, A., MacPhail, S. H., Johnston, P. J., Banath, J. B. and Olive, P. L. (2000). Changes in subcellular distribution of topoisomerase II alpha correlate with etoposide resistance in multicell spheroids and xenograft tumors. *Cancer Res.* **60**, 5747-5753.

Ossareh-Nazari, B., Bachelerie, F. and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278**, 141-144.

Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M. and Görlich, D. (1998). NTF2 mediates nuclear import of Ran. *EMBO J.* **17**, 6587-6598.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T. and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J.* **17**, 554-564.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* **4**, 635-651.

Siomi, M. C., Eder, P. S., Kataoka, N., Wan, L., Liu, Q. and Dreyfuss, G. (1997). Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J. Cell Biol.* **138**, 1181-1192.

Sodek, J., Hodges, R. S., Smillie, L. B. and Jurasek, L. (1972). Amino-acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure. *Proc. Natl. Acad. Sci. USA* **69**, 3800-3804.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J. and Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J.* **18**, 1660-1672.

Sullivan, D. M., Latham, M. D. and Ross, W. E. (1987). Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse, and Chinese hamster ovary cells. *Cancer Res.* **47**, 3973-3979.

Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. M., Wang, J. Y. and Hope, T. J. (1998). Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **95**, 7457-7462.

Valkov, N. I., Gump, J. L. and Sullivan, D. M. (1997). Quantitative immunofluorescence and immunoelectron microscopy of the topoisomerase II alpha associated with nuclear matrices from wild-type and drug-resistant Chinese hamster ovary cell lines. *J. Cell Biochem.* **67**, 112-130.

Valkov, N. I., Gump, J. L., Engel, R. and Sullivan, D. M. (2000). Cell density-dependent VP-16 sensitivity of leukaemic cells is accompanied by the translocation of topoisomerase II alpha from the nucleus to the cytoplasm. *Br. J. Haematol.* **108**, 331-345.

Van den Hoff, M. J., Moorman, A. F. and Lamers, W. H. (1992). Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res.* **20**, 2902.

Wada, A., Fukuda, M., Mishima, M. and Nishida, E. (1998). Nuclear export of actin: a novel mechanism regulating the subcellular localization of a major cytoskeletal protein. *EMBO J.* **17**, 1635-1641.

Wang, J. C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nat. Rev. Mol. Cell. Biol.* **3**, 430-440.

Watt, P. M. and Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *Biochem. J.* **303**, 681-695.

Weis, K. (2003). Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* **112**, 441-451.

Wen, W., Meinkoth, J. L., Tsien, R. Y. and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463-473.

Wessel, I., Jensen, P. B., Falck, J., Mirski, S. E., Cole, S. P. and Sehested, M. (1997). Loss of amino acids 1490Lys-Ser-Lys1492 in the COOH-terminal region of topoisomerase II alpha in human small cell lung cancer cells selected for resistance to etoposide results in an extranuclear enzyme localization. *Cancer Res.* **57**, 4451-4454.

Wu, C. H., Huang, H., Arminski, L., Castro-Alvear, J., Chen, Y., Hu, Z. Z., Ledley, R. S., Lewis, K. C., Mewes, H. W., Orcutt, B. C., et al. (2002). The Protein Information Resource: an integrated public resource of functional annotation of proteins. *Nucleic Acids Res.* **30**, 35-37.

Yoneda, Y., Hieda, M., Nagoshi, E. and Miyamoto, Y. (1999). Nucleocytoplasmic protein transport and recycling of Ran. *Cell Struct. Funct.* **24**, 425-433.

Zolotukhin, A. S. and Felber, B. K. (1997). Mutations in the nuclear export signal of human ran-binding protein RanBP1 block the Rev-mediated posttranscriptional regulation of human immunodeficiency virus type 1. *J. Biol. Chem.* **273**, 11356-11360.