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Fragment responsible for translocation in the N-terminal domain of human topoisomerase I

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Abstract

The N-terminal domain is a fragment that binds proteins and anchors topoisomerase I in the nucleolus. As a separate polypeptide, it translocates from the nucleolus to nucleoplasm upon camptothecin treatment. In this paper, we show that the translocation depends on the short fragment of the domain (residues from 1 to 67). We also present a list of proteins that specifically bind to the fragment responsible for translocation.

Keywords: Topoisomerase I; N-terminal domain; Translocation; Protein binding

Human topoisomerase I (topo I) is the main enzyme responsible for DNA relaxation [1,2] and a protein kinase specific for serine/arginine motifs present in essential splicing factors [3]. It is also a sole cellular target for anticancer drugs derived from the plant alkaloid camptothecin (CPT) [4]. The latter activity results from CPT-induced stabilization of the transient cleavage introduced by topo I in the course of catalyzing of DNA relaxation, which can be further processed into double DNA break [1].

Distribution of topo I among subnuclear compartments decides which nuclear process engages the activities of the enzyme. It also defines DNA regions which are damaged upon CPT treatment [5]. In the interphase cells large majority of topo I is concentrated in the nucleolus and exhibits the preference for fibrillar centers of nucleoli [6]. The fine nucleolar localization results from higher mobility of topo I in the nucleoplasm than in the nucleolus, and from docking of the N-terminal domain of topo I at the nucleolar fibrillar centers [6]. It is reflected in behavior of topo I in cells treated with CPT. The treatment results in translocation of topo I from the nucleolus into the nucleoplasm [7,8]. The movement exhibits a dual kinetics with a rapid (~1 min) and a slow phase (~20 min), depending on the form of topo I [9]. If the active form is used, a rapid translocation is observed which has been suggested to result from the retardation of topo I mobility in the nucleoplasm that leads to recovery of the balance of concentrations between the nucleolus and the nucleoplasm [9]. If the inactive N-terminal domain is analyzed, a slow translocation takes place [9–11] which is thought to be backed by changed interactions of topo I with a molecular partner(s) [9], most possibly protein(s) [10]. It is considered that the interaction of topo I with the protein partners could be modified by its sumoylation which accompany the CPT-induced translocation [12]. However, conflicting results have been presented on the role of sumoylation in the nucleolar depletion of topo I [9,13].

Topo I is a single polypeptide of 765 amino acid residues composed of four domains called: N-terminal (residues 1–214), core (residues 215–635), linker (636–712), and C-terminal (713–765) domain [1]. The core domain is further divided into three subdomains: the cap region, including subdomains I and II (residues 215–433) and the subdomain

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III (residues 434–635). In the previous work [14–16] we identified the N-terminal domain and the cap as main regions of topo I that bind proteins. Looking for a possible role of the protein: protein interactions in distribution of topo I among subnuclear compartments we studied in this paper CPT-induced translocation from the nucleolus to the nucleoplasm for the protein binding fragments of the protein. We identified a fragment responsible for the translocation and a set of proteins which bound to this fragment.

Materials and methods

Plasmids construction and site-directed mutagenesis. The coding sequences of topo I or topo I fragments were generated by PCR from the full length cDNA of topo I. The primers and restriction endonucleases used to digest the PCR fragments are shown in Table 1.

Plasmids for HeLa transfection were constructed on the basis of pEGFP-N1 vector (Clontech). Kozak sequence and HA epitope sequence were added at the N-terminus of each protein. Moreover, artificial NLS sequence from SV40 large T antigen [17] was added to the proteins except for constructs containing full length N-terminal domain of topo I (full length topo I and topo[1–214], topo[1–433] fragments). NLS, HA epitope, and Kozak sequence were also added to EGFP in pEGFP-N1 vector. Plasmids carrying sequences coding baits for affinity chromatography were constructed on the basis of pGEX-4T-1 (Amersham).

Mutations corresponding to amino acid substitutions. K103R, K117R, and K153R were introduced sequentially by DpnI-mediated site-directed mutagenesis, using QuikChange Site-Directed Mutagenesis Kit, as described by the manufacturer (Stratagene). The primers used are shown in Table 1.

All the obtained clones were subjected to nucleotide sequencing to exclude possible errors introduced during PCR.

HeLa cells transfection and drug treatment. Transfection of the HeLa cells was performed with FuGENE HD reagent (Roche Applied Science) according to manufacturer instruction. Twenty-four hours after transfection cells were subjected to the CPT treatment. CPT dissolved in DMSO was added to the medium to the final concentration of 25 μM for 40 min. Final concentration of DMSO was 1%. Cells were washed with PBS and observed under laser scanning confocal microscope LSM510 (Zeiss) without any fixing.

Affinity chromatography. GST and two GST-fusion proteins containing topo[1–67] or topo[68–214] fragment of human topo I polypeptide were prepared in *Escherichia coli* strain BL21(DE3) (Novagen) using pGEX-4T-1 vector. Affinity chromatography was performed as described previously [14] with some modifications. GST or GST-fusion proteins bound to glutathione–agarose beads were incubated for 2 h with the HeLa nuclear extract and then the baits were released by digestion with thrombin. Proteins were analyzed by trypsinization, microcapillary HPLC and electrospray ionization quadruple time-of-flight MS analysis performed at Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics PAS, Warsaw. The mass spectrometry data were used for identification of proteins with the Mascot program (http://www.matrixscience.com) [18].

Results

Mapping of the region of topo I responsible for protein binding-dependent translocation

Transfection of mammalian cells with vectors coding the fragments of topo I that lack NLS leads to cytoplasmic localization of the recombinant proteins [19]. Because the nucleolar/nuclear partitioning of such prepared fragments cannot be studied, we have added the NLS coming from SV40 large T antigen [17] to each fragment used in this work except for polypeptides comprising full length N-terminal domain of topo I which carries natural NLS sequences. NLS was also added to the EGFP coded by pEGFP-N1 vector. As it can be seen for the EGFP, the NLS directed major part of the polypeptide to the nucleus but not to the nucleolus (Fig. 1). The fluorescence was evenly distributed throughout the nucleoplasm and its small amount was also visible in the cytoplasm. The distribution was not sensitive to the CPT treatment (Fig. 1).

To study a protein binding-dependent translocation, a fragment topo[1–433] was used which comprised the two regions of topo I involved in proteins binding [14]. Its distribution in CPT treated cells was different from that of the complete topo I (Fig. 1). Delocalized topo[1–433] did not spread throughout the nucleoplasm but accumulated in the nuclear body-like structures.

To ascribe the effect of delocalization to one of two regions that bind proteins we divided the initial construct into two parts: the N-terminal domain topo[1–214] and the cap region topo[215–433]. Only the N-terminal domain was concentrated in the nucleolus and translocated from it upon CPT treatment (Fig. 1). In contrast to the above, the cap region was uniformly distributed in the nucleus and its distribution did not change in CPT treated cells (Fig. 1). This observation was in agreement with previous reports that the N-terminal fragment solely moves out from the nucleoli with a slow kinetics upon CPT treatment [9–11]. It also excludes any role of the cap region in the translocation.

Effects of sumoylation on the translocation of the N-terminal domain

Because of conflicting results published on the role of sumoylation in the translocation of both the complete topo I [9,13] and the N-terminal domain [9,11], we tested here the K → R mutants of the topo[1–214] fragment, unable to be sumoylated, in the system employed by us. Sumoylation occurs at three sites in the N-terminal domain: K103, K117, and K153 [13], with the major site K117 shown to be polysumoylated [12]. The pictures observed here for the K → R mutants at each sumoylated site of the N-terminal domain, both in the untreated and CPT treated cells, did not significantly differ from the wild topo[1–214] fragment (not shown). In the CPT treated cells, a triple mutant topo[1–214]K103RK117RK153R moved out from the nucleoli (Fig. 2), similarly as the wild protein. Therefore, sumoylation sites did not mark out borders for the translocation fragment of the N-terminal domain.

Delineation of the translocation fragment

To delineate the region of the N-terminal domain that supported the translocation we further divided topo[1–214] into smaller fragments and tested their behavior upon

Table 1
Primers used in the study

Protein	Primers	Restriction endonucleases
HeLa transfection		
NLS-EGFP	5′-CCCAAGCTTGCCGCCACCATG-3′ 5′-ATGCGGATCCACCTTTCTCTTCTTTT TTGGATCTGCGTAGTCTGGCACGTC-3′	HindIII/BamHI
topo[1–765]	5′-CATGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAATGAGTGGGGAC CACCTCC-3′ 5′-GATGGATCCGCAAACTCATAGTCTTCATCAG CC-3′	EcoRI/BamHI
topo[1–433]	5′-CATGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAATGAGTGGGGAC CACCTCC-3′ 5′-GTGTGGATCCCGTGAACTAGGGTTAAGCATG-3′	EcoRI/BamHI
topo[1–214]	5′-CATGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAATGAGTGGGGAC CACCTCC-3′ 5′-GCATGTCGACACGCCTTCAGGATAGCGCTCT-3′	EcoRI/SalI
topo[215–433]	5′-CTGTGTCGACGATCAAGTGGAAATTCCTAGA AC-3′ 5′-GTGTGGATCCCGTGAACTAGGGTTAAGCATG ATG-3′	SalI/BamHI
topo[1–133]	5′-CATGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAATGAGTGGGGAC CACCTCC-3′ 5′-CATGTCGACACCTTTCTCTTCTTTTTTGGATC TATATCCTCTTTAGGAGGAAC-3′	EcoRI/SalI
topo[134–214]	5′-CATGAATTCGCCGCCACCATGGACTACCCAT ACGACGTGCCAGACTACGCAAAGCCATTAAAG AGACCTCG-3′ 5′-CATGTCGACACCTTTCTCTTCTTTTTTGGATC GCCTTCAGGATAGCGCTCT-3′	EcoRI/SalI
topo[1–67]	5′-CATGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAATGAGTGGGGAC CACCTCC-3′ 5′-CATGTCGACACCTTTCTCTTCTTTTTTGGATCGGTCTTCTCCTTCTCTTTGT-3′	EcoRI/SalI
topo[68–133]	5′-CTGAATTCGCCGCCACCATGGACTACCCATA CGACGTGCCAGACTACGCAAAACACAAAGATG GAAGCTC-3′ 5′-CATGTCGACACCTTTCTCTTCTTTTTTGGATCT ATATCCTCTTTAGGAGGAAC-3′	EcoRI/SalI
topo[134–170]	5′-CATGAATTCGCCGCCACCATGGACTACCCAT ACGACGTGCCAGACTACGCAAAGCCATTAAAG AGACCTCG-3′ 5′-CGGGTCGACACCTTTCTCTTCTTTTTTGGATCCTCTTCTTCTTCTAGTTT-3′	EcoRI/SalI
topo[171–214]	5′-CGGAATTCGCCGCCACCATGGACTACCCATACGACGTGCCAGACTACGCAGGTAAATTGAAAA AACCCAAG-3′ 5′-CATGTCGACACCTTTCTCTTCTTTTTTGGATC GCCTTCAGGATAGCGCTCT-3′	EcoRI/SalI
Affinity chromatography		
topo[1–67]	5′-CATGGATCCATGAGTGGGGACCACCTC-3′ 5′-CATGTCGACTAGGTCTTCTCCTTCTCTTTGT-3′	BamHI/SalI
topo[68–214]	5′-GCTCGGATCCAAACACAAAGATGGAAGCTC-3′ 5′-CCTGTCGACTTAGCCTTCAGGATAGCGCTC-3′	BamHI/SalI
Mutagenesis		
K103R	5′-CCTCTGGGGATGCAAAAATACGGAAGGAGAAGGAAAATGGC-3′ 5′-GCCATTTTCCTTCTCCTTCCGTATTTTTGCATCCCCAGAGG-3′	
K117R	5′-CTCTAGTCCACCACAAATTCGCGATGAACCTGAAGATGATGG-3′ 5′-CCATCATCTTCAGGTTCATCGCGAATTTGTGGTGGACTAGAG-3′	
K153R	5′-GATGTTGATTATAAACCTAAGAAAATTCGCACAGAAGATACCAAGAAGGAGAAG-3′ 5′-CTTCTCCTTCTTGGTATCTTCTGTGCGAATTTTCTTAGGTTTATAATCAACATC3′	

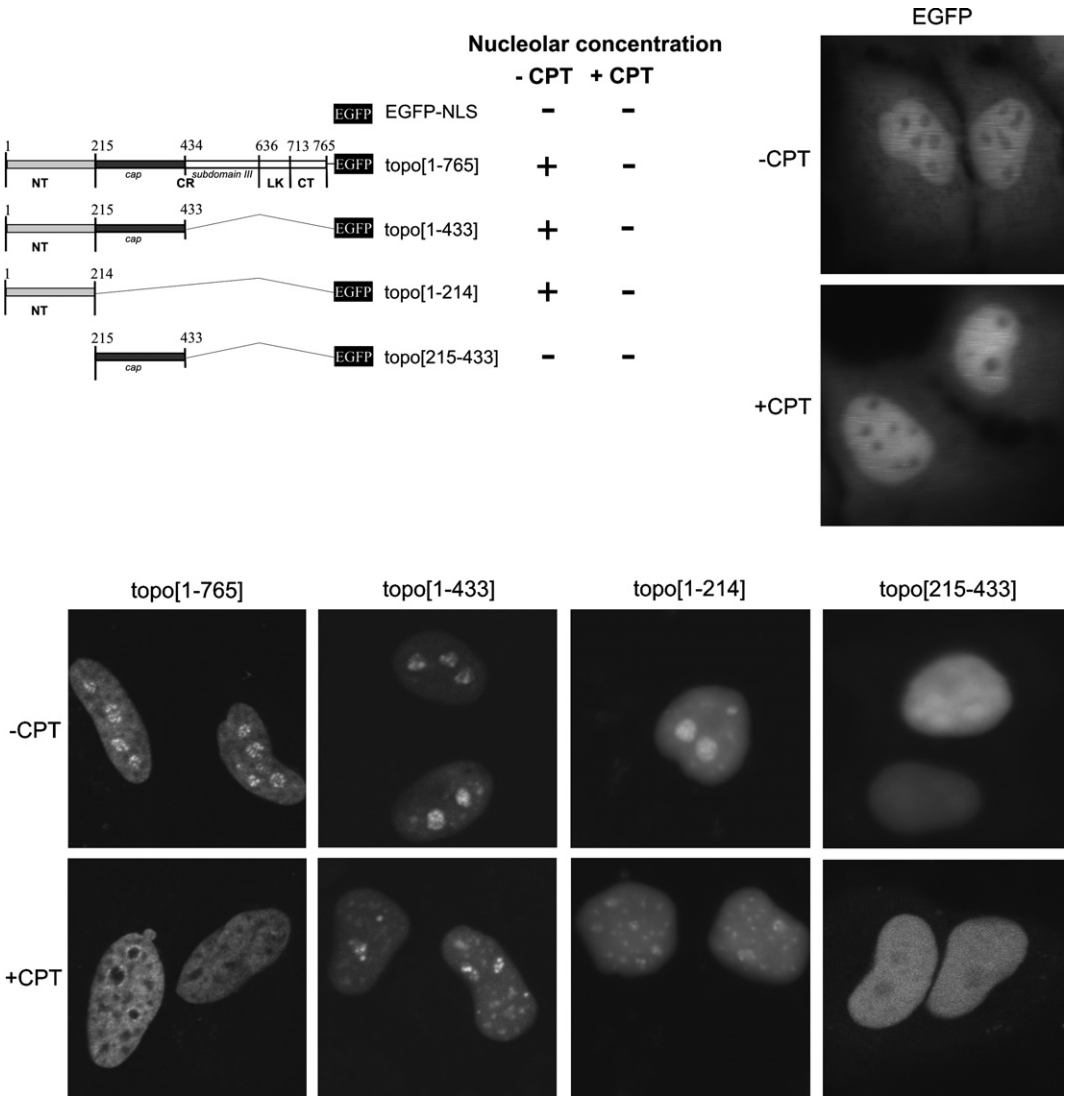

Fig. 1. Nuclear distribution of the complete topo I and its protein binding domains without and with CPT treatment.

CPT treatment. It was possible to estimate the subnuclear localization for fragments as short as several dozens of amino acids. However, pictures obtained in those cases was obviously less clear than for the topo[1–214] fragment (Fig. 3). If shorter fragments of the N-terminal domain were analyzed, the fine distribution of the fluorescence differed depending on the length of the polypeptide. Thus, we recognized that nucleolar delocalization appeared when fluorescence grew up in nucleoplasm and went down in nucleoli comparing to control cells even if traces of fluorescent polypeptide were still visible inside the nucleoli.

The experiments indicated preferential nucleolar localization of two fragments: topo[1–67] and topo[171–214]. Lack of nucleolar accumulation of topo[68–133] and topo[134–170] was not due to separation of the possible nucleolar localization signal into two parts because neither topo[68–170] was accumulated in the nucleolus (not shown). Out of two fragments localized in the nucleolus only topo[1–67] moved out from the nucleolus upon CPT treatment (Fig. 3C). Shorter fragments of topo I did not accumulate effectively in nucleoli (not shown).

The above experiments indicate that translocation fragment in the N-terminal domain includes the first 67 residues of topo I. In agreement with experiments described in the previous section, the translocation fragment does not contain sumoylation sites [13].

Proteins binding to the topo[1–67] fragment

To identify proteins that bound to the fragment responsible for CPT-induced delocalization we performed affinity chromatography with GST-fused topo[1–67] and topo[68–214] baits, and analyzed the proteins by MS. A list of proteins bound to each bait is presented in Table 2. Each experiment was performed in duplicate and only the proteins identified twice with the value of the total score (according to the Mascot program [18]) higher than 100 are listed.

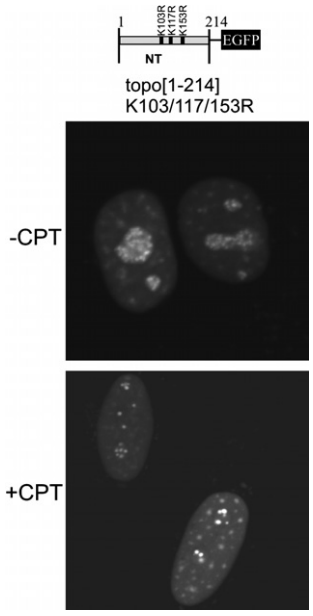

Fig. 2. Lack of the effect of sumoylation on translocation of the N-terminal domain upon CPT treatment. Nuclear distribution of the N-terminal domain mutated at all sumoylation sites is shown.

Large number of proteins presented in Table 2 was previously identified as binding to the bait containing the complete N-terminal domain [14]. Several other are new because they were previously identified with the value of the total score too low to be considered as interacting with the complete N-terminal domain [14]. Several proteins unspecifically associated with both baits, e.g. nucleolin, hnRNP A1, hnRNP A2/B1, treacle (TCOF 1) or NF-45 protein. However, several other selectively bound to the [1–67] bait. Among the latter group, we found SR proteins: SRp40 (SFRS5), hTra2b (SFRS10), and SF2/ASF (SFRS1). Moreover, another SR protein SRm300 which indeed bound to both baits, was identified because of 19 and 22 peptide hits (values for two independent experiments) in the case of topo[1–67] but only because of 3 and 8 peptide hits in the case of topo[68–214]. Taking together, we concluded that specific interaction with SR proteins distinguished binding properties of topo[1–67] from the remaining fragment of the N-terminal domain.

Besides SR proteins, also several other proteins specifically recognized the topo[1–67] bait. They were: Acinus,

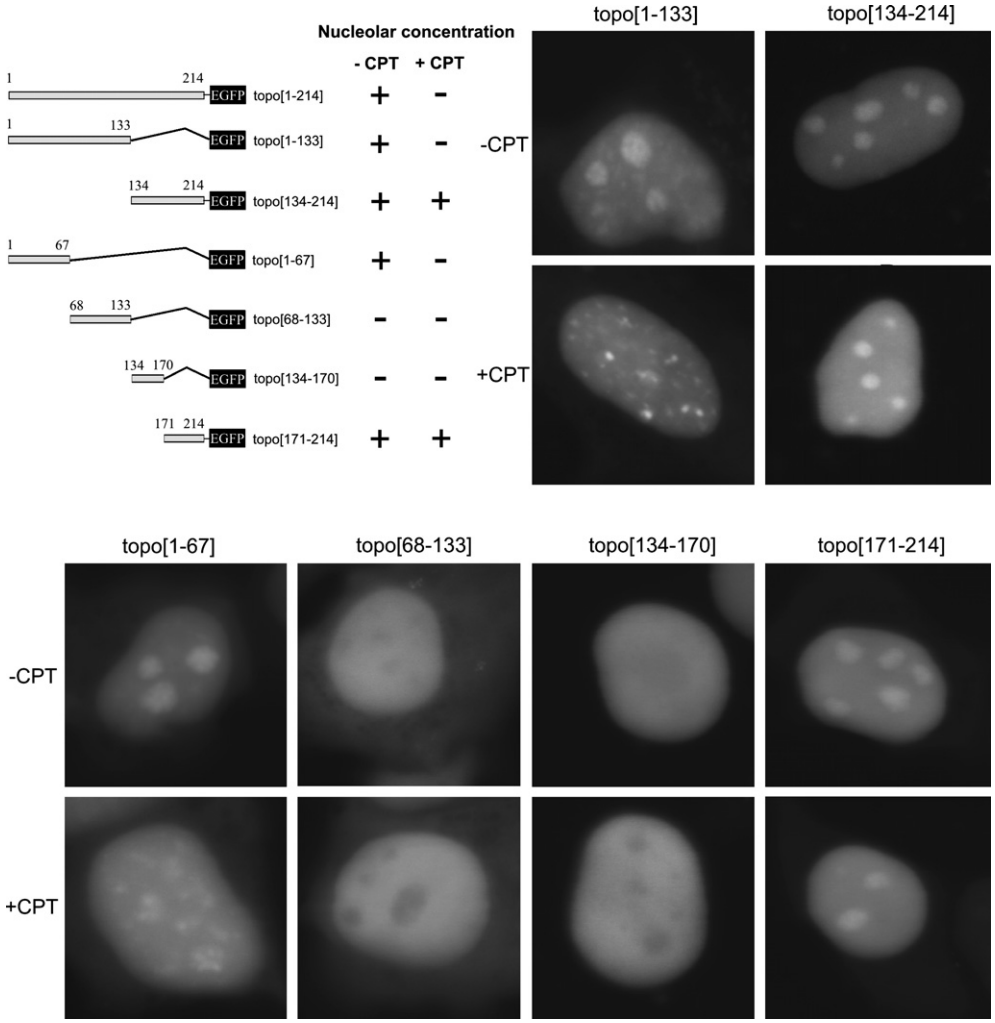

Fig. 3. Mapping of the region of the N-terminal domain responsible for protein binding-dependent translocation upon CPT treatment.

Table 2
The lists of proteins that associate with topo[1–67] or topo[68–214] fragments. Proteins that specifically associate with each fragment are underlined

topo[1–67]			topo[68–214]		
Protein	Accession No.	Score[a]	Protein	Accession No.	Score[a]
SRm300	Q9UQ35	694	hnRNP A1	P09651	555
Nucleolin	P19338	301	Nucleolin	P19338	349
Treacle (TCOF1)	Q13428	300	PARP-1	P09874	341
PSF	P23246	293	PSF	P23246	336
hnRNP A1	P09651	263	hnRNP A2/B1	P22626	284
Acinus	Q9UKV3	224	Treacle (TCOF1)	Q13428	222
RNA helicase p72	Q92841	193	hnRNP U	Q00839	214
RNA helicase A	Q08211	184	hnRNP D	Q14103	199
RNA helicase p68	P17844	178	RNA helicase p68	P17844	195
RNA helicase II/Gu	Q9NR30	175	RNA helicase p72	Q92841	194
hnRNP A0	Q13151	169	p54nrb	Q15233	193
hnRNP A2/B1	P22626	167	hnRNP K	P61978	189
SRp40 (SFRS5)	Q13243	161	THOC4(Aly/REF)	Q86V81	189
Trap150	Q9Y2W1	140	hnRNP A0	Q13151	171
hnRNP U	Q00839	137	NF 45	Q12905	152
Prp8 (U5-220kD)	Q6P2Q9	136	hnRNP AB	Q99729	147
hnRNP R	O43390	135	B23	P06748	142
PARP-1	P09874	131	hnRNP Q	O60506	138
hnRNP AB	Q99729	127	SRm300	Q9UQ35	134
NF 45	Q12905	126	hnRNP R	O43390	129
Parafibromin	Q6P1J9	120	PRP19/PSO4	Q9UMS4	124
Ctr9	Q6PD62	116	IFI-4 deaminase	P55265	120
hnRNP A3	P51991	114	RNA helicase A	Q08211	116
p54nrb	Q15233	114	Ki-67	P46013	115
Paf1	Q9H166	111	CSL4	Q9Y3B2	113
hnRNP Q	O60506	109	hnRNP A3	P51991	108
SF2/ASF (SFRS1)	Q07955	107	HMG I(Y)	P17096	103
HTra2b (SFRS10)	P62995	105			
THOC4(Aly/REF)	Q86V81	103			
PRP19/PSO4	Q9UMS4	100			
Ki-67	P46013	100			

[a] The average score value from two experiments.

Ctr9, Paf1, parafibromin, Prp8, RNA helicase II/Gu, and Trap150 (Table 2).

Discussion

This study presents two findings that concern a role of protein:protein interactions in the nucleolus/nucleoplasm partition of topo I. The first finding points to two fragments, topo[1–67] and topo[171–214], which can preferentially localize in the nucleolus and shows that the first fragment loses this feature in CPT treated cells. The other finding is that a set of proteins specifically interact with the topo[1–67] fragment.

The motif $(R/K)(R/K)\times(R/K)$ has been defined as a possible nucleolar localization sequence (NoLS) for several human proteins [20,21]. Several sequences that fulfill the above consensus can be found in the N-terminal domain of topo I. A role of separate regions of the N-terminal domain in its subcellular localization has been studied by Mo et al. [19]. They revealed a sequence active in the nucleolar localization of the N-terminal domain which was between aa 188 and 199. This region is included in the fragment topo[171–214], found in this work as preferentially localized in the nucleolus. Most possibly, the NoLS is either ^{191}KKKK^{194} or ^{193}KKPK^{196}, the two motifs that fulfill the consensus for the nucleolar targeting and are present in the fragment topo[171–214].

In the study mentioned above [19], the fragments lacking a functional nuclear localization signal (NLS), which has been ascribed to the sequence between aa 150 and 156, were found in cytoplasm and thus missing from the analysis of the nucleolar/nuclear partition. However, in the complete topo I these fragments are targeted to the nucleus because of NLS(s) present in other regions of the protein. In keeping with the above, we fused the analyzed fragments to the artificial NLS. Due to this strategy we revealed another fragment, topo[1–67], active in the nucleolar targeting of the N-terminal domain. There are two sequences in the topo[1–67] fragment that fulfill the NoSL consensus: ^{39}KKEK^{42} and ^{59}KKHK^{62}.

A unique feature of the topo[1–67] fragment is that it loses its nucleolar localization in CPT treated cells. Proteomic analysis of the nuclear proteins that specifically bound to the fragment did not give an answer to the question of the direct reason of the translocation because majority of these proteins are not of nucleolar origin. Most of them have been previously found in the newly spliced mRNPs isolated from the nuclear extract of HeLa cells [22]. They

are: Acinus, Trap150, Prp8, and SR proteins. Proteins that compose mRNPs are also found among those interacting with both topo[1–67] and topo[68–214] fragments. They are: hnRNP proteins A0, A1, A2/B1, A3, Q, R, and U; RNA helicases p68 and A (NDH II); NF 45 and THOC4 (Aly/REF). The above observation is consistent with the suggested role for topo I in splicing regulation [23]. Another three of the proteins that specifically bound to the topo[1–67] fragment are Paf1, parafibromin, and Ctr9. All they are constituents of the Paf1 complex [24] which associates with the RNA II polymerase [25] and is linked with methylation of histones H3 and H4 [26]. This observation could be relevant to localization of topo I at the transcription active regions which is linked with its N-terminal domain [27,28]. The only nucleolar protein that specifically bound to the [1–67] fragment was RNA helicase II/Gu. However, topo I and RNA helicase II/Gu localize to different regions of the nucleolus: to the fibrillar center [6] and to the granullar component [29], respectively.

In summary, proteins listed as specifically interacting with the topo[1–67] fragment of topo I could be rather linked with targeting of topo I to processes that are carried out in the nucleoplasm than with anchoring it in the nucleolus. Therefore, proteins linked with the latter process should be rather searched among those that bind both with the topo[1–67] fragment and the remaining part of the N-terminal domain. Based on localization experiments, RNA polymerase I has been suggested to play such a role [6]. We did not find RNA polymerase I among proteins interacting with the topo[1–67] fragment nor any other fragment of topo I [14]. On the other hand, treacle protein which bound both to the topo[1–67] and topo[68–214] fragments but not to any other fragment of topo I [14] has been shown to interact with UBF [30], a protein directly associated with RNA polymerase I [31].

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