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Abstract

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POLY(ADP-RIBOSE) BINDS TO THE SPLICING FACTOR ASF/SF2 AND REGULATES ITS PHOSPHORYLATION BY DNA TOPOISOMERASE I

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Running head: Poly(ADP-ribose) regulates topoisomerase I functions

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Human DNA topoisomerase I plays a dual role in transcription, by controlling DNA supercoiling and by acting as a specific kinase for the SR-protein family of splicing factors. The two activities are mutually exclusive, but the identity of the molecular switch is unknown. Here we identify poly(ADP-ribose) as a physiological regulator of the two topoisomerase I functions. We found that in the presence of both DNA and the alternative splicing factor/splicing factor 2 (ASF/SF2, a prototypical SR-protein), poly(ADP-ribose) affected topoisomerase I substrate selection and gradually shifted enzyme activity from protein phosphorylation to DNA cleavage. A likely mechanistic explanation was offered by the discovery that poly(ADP-ribose) forms a high affinity complex with ASF/SF2 thereby leaving topoisomerase I available for directing its action onto DNA. We identified two functionally important domains, RRM1 and RS, as specific poly(ADP-ribose) binding targets. Two independent lines of evidence emphasize the potential biological relevance of our findings: i) in HeLa nuclear extracts, ASF/SF2- but not histone- phosphorylation was inhibited by poly(ADP-ribose); ii) an in silico study based on gene expression profiling data revealed an increased incidence of alternative splicing within a subset of inflammatory response genes that are dysregulated in cells lacking a functional poly(ADP-ribose) polymerase-1. We propose that poly(ADP-ribose) targeting of topoisomerase I and ASF/SF2 functions may participate in the regulation of gene expression.

DNA topoisomerase I (topo I) is a constitutively expressed multifunctional enzyme that localizes at active transcription sites (1,2). Its best known function is to control the topological state of DNA by relieving torsional stress that is generated following DNA strand separation during transcription, replication, repair (3-5). The catalytic mechanism involves the formation of a DNA-topo I complex (cleavage complex) with the enzyme being covalently bound to the 3'-end of the cleaved DNA strand through a tyrosine-phosphate ester bond. Cleavage complexes are usually short-lived; their stabilization by compounds of the camptothecin (CPT) family of anticancer drugs may cause DNA strand break accumulation and eventually lead to cell death. Human topo I can relax both negative and positive supercoils by controlled rotation of the DNA strand downstream of the cleavage site followed by break resealing and restoration of an intact DNA duplex.

In addition to relaxing supercoiled DNA, human topo I also plays a major role in pre-mRNA splicing, being endowed with a protein kinase activity targeted at a group of splicing factors of the Serine-Arginine (SR) rich protein family (6). SR-proteins function both as components of the basal RNA splicing machinery and as regulators of alternative splicing (7,8). Moreover, SR-proteins are involved in the control of mRNA transport and stability (8,9) and contribute to the maintenance of genomic stability (10). SR-proteins are structurally characterized by having one or two domains that include a RNA Recognition Motif (RRM) at the N-terminus, and a Serine-Arginine rich C-terminal domain (RS domain) containing a variable number of SR dipeptidic repeats. Phosphorylation at Serine residues in such sequences regulates SR-protein functions as well as their subnuclear localization (7-9,11,12). Besides topo I, other kinases are also involved in SR-protein phosphorylation; these include the SR-protein specific kinases 1 and 2 (SRPK1 and SRPK2) and the cell cycle-dependent dual specificity kinase Ctk/Sty (13). Moreover, Akt/protein kinase B phosphorylation of SR-protein family members appears to play a critical role in signal transduction pathways linking extracellular stimuli (hormones, mitogens) to changes in gene expression via regulation of...
alternative splicing and mRNA translation (14,15). Topo I depletion results in the hypophosphorylation of SR-proteins and impaired exonic enhancer-dependent splicing (16). Likewise, inhibition of topo I-dependent SR-protein phosphorylation by indolocarbazole antitumor drugs has been shown to interfere with the spliceosome assembly pathway, leading to altered gene expression and eventually to cell death (17).

Thus, human topo I may either bind DNA and catalyze its relaxation, or bind SR-proteins and ATP and play the role of a kinase. The two activities are mutually exclusive and they are most likely the functional expression of distinct conformational states (18). What regulates such structural and functional transitions is unknown.

Human topo I is a member of the poly(ADP-ribose)-binding family of proteins (19). Poly(ADP-ribose) (PAR) is the product of a class of enzymes known as PAR polymerases (PARPs) (20). PARPs utilize NAD+ as a source of ADP-ribose units and catalyze the covalent modification of a number of proteins (heteromodification), including themselves (automodification), with an array of linear or branched ADP-ribose chains of variable lengths; these polymers are then degraded by a specific PAR glycohydrolase (PARG), thus making the reaction reversible (20,21). PARP-1 and PARP-2, the best known members of the PARPs' family, depend on DNA strand breaks for activity and are responsible for most PAR synthesized in the nucleus of eukaryotic cells both under physiological and DNA damage conditions (20,21). Protein targeting by PARP-bound polymers via non-covalent, yet specific interactions, is emerging as an important regulatory mechanism for diverse biological functions, including transcription, DNA damage signaling and checkpoint activation, proteasomal histone degradation and mitotic spindle formation (22,23). Topo I bears three PAR-binding sites localized in domains that are critical for the catalytic activity of the enzyme on DNA and for its regulation (24). In fact, PAR has a dual effect on topo I: it inhibits DNA cleavage (thus preventing initiation of new catalytic cycles), while it stimulates the religation activity of the enzyme blocked in a ternary complex with nicked DNA and CPT (thus counteracting the poisoning effect of the drug) (24).

In this study we addressed the question whether PAR could also affect topo I kinase activity and/or act as a molecular switch of distinct topo I functions. ASF/SF2, a prototype of the SR-protein family, was used as a specific substrate for the topo I kinase activity.

**MATERIALS AND METHODS**

Purified human topo I was obtained from TopoGen. This enzyme undergoes spontaneous conversion into a 70 kDa form lacking the N-terminal domain (ΔN-topo I). Full length Histagged human topo I expressed in a baculovirus system was from Jena Bioscience (distributed by Alexis). Topo I homogeneity was >95%. GST-SRPK1 was from Upstate. Highly purified recombinant PARP-1 and PARP-2 were purchased from Alexis.

Protein-free, affinity purified [14C]-poly (ADP-ribose) (PAR), [32P]-PAR and [32P]-5'-end labeled ds-oligonucleotide, used as a topo I substrate in DNA cleavage assays, were prepared as previously described (24).

All experiments were repeated at least three times and confirmed with different preparations of topo I, SR-proteins, PAR.

Recombinant proteins - Human topo I was expressed in *Saccharomyces cerevisiae EKY3* strain and purified essentially as described by Kowalska-Loth et al (25). Briefly, the expression of topo I was induced with 2% galactose. After 4 hours, cells were harvested, washed once with SED buffer (1 M sorbitol, 25 mM EDTA, pH 8.0, 50 mM dithiotreitol (DTT) and treated with 5 mg/ml zymolyase 100T (Seikagaku) for 30 min at 30 °C. Spheroplasts were pelleted by centrifugation and extracted twice with YLS buffer (20 mM Tris, pH 7.5, 0.5 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF). Yeast extracts were adjusted to buffer conditions (0.35 M NaCl, 25 mM Tris-HCl pH 7.5, 3 mM MgCl2, 10 mM β-mercaptoethanol, 10% glycerol, 1mM PMSF), loaded onto heparin-agarose column (Bio-Rad) and eluted by increasing salt concentration. Topo I containing fractions were pooled and loaded onto a Ni-NTA agarose column (Qiagen) for further purification. Finally, purified topo I was concentrated on Biomax-5K columns (Millipore).
His-tagged SF2 was expressed in *E. coli* TG1 strain and purified as described by Rossi et al (6).

GST-tagged SF2 and SF2 fragments (GST-ASF/SF21-119, GST-ASF/SF2120-194, GST-ASF/SF2195-248) were expressed in *E. coli* strain BL21(DE) (Novagen) and purified on glutathione-agarose (Sigma) as previously described (25). GST-ASF/SF2195-248 was subjected to further purification on heparine-agarose column after dilution in 50mM Tris-HCl buffer, pH 8.0, containing, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM EDTA, 1mM PMSF. Elution was carried out in the same buffer with increasing NaCl concentrations (0.1-1 M). Purified polypeptides were concentrated on Biomax-5K columns (Millipore) and buffer was exchanged to 20 mM HEPES, pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05 % Triton X-100, 10 % glycerol, 1 mM DTT, 1 mM PMSF.

**Topo I activity assays** - Topo I DNA cleavage activity was assayed using a \([\text{32P}]\)-5’-end labelled ds-oligonucleotide containing a single topo I binding/cleavage site, as previously reported (24). Reaction mixtures (15 µl) were assembled on ice and contained 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, 20 µM CPT, 0.2 mg/ml BSA, 2% glycerol, human topo I (0.2-0.27 pmol). Reaction was started by addition of [\text{32P}]-5’-end labeled ds-oligonucleotide (0.02-0.04 pmol; 0.5-1.1x10^6 dpm/pmol) and carried on for 10-20 min at 37 °C. After termination by addition of concentrated Laemmli buffer, cleavage complexes were separated by SDS-PAGE on 7.5% polyacrylamide gels and visualized by autoradiography. When present, affinity purified PAR or ASF/SF2 were added to the reaction mixture at the amounts indicated in the Figures.

**PAR binding assay** - The PAR binding assay was carried on essentially as described by Panzeter et al (26). Proteins were immobilized on nitrocellulose either by western blotting after electrophoretic separation on polyacrylamide gels, or by slot blotting. Duplicate samples were either gold/Coomassie blue stained for protein visualization or probed with [\text{32P}]-PAR, washed with 10 mM Tris-HCl, 0.15 M NaCl, 0.05% (v/v) Tween-20, pH 7.4 (TBST) and analyzed by autoradiography. Where indicated, 0.5 M NaCl was added to the washing buffer (0.5 M NaCl-TBST).

**Protein extracts** - HeLa S3 cells were cultured in complete DMEM under standard culturing conditions and harvested at a subconfluent stage to be used for nuclear and cytoplasmic protein extract preparations, as previously described (27).

**Quantitative analyses** - Autoradiographic bands were quantified by scanning densitometry using the GS-710 Bio-Rad densitometer and the image analysis software QuantityOne (Bio-Rad). Data are expressed as mean of at least 3 independent experiments +/- standard deviation (SD).

**Bioinformatics** - Peer-reviewed literature was surveyed for comparative genome-wide studies.
of the transcriptomes of parp-1 knockout mammalian cells and their wild type counterparts, both under physiologic conditions and after exposure to cytotoxic stimuli. Then, gene expression profiling data were matched against the Alternative Splicing Annotation Project (ASAP II) database (28). Only genes coding for at least one expressed alternatively spliced isoform were taken as positive and expressed as percent of total analyzed genes.

RESULTS

Topo I protein kinase activity is inhibited by PAR. In a reconstituted system consisting of PAR, topo I and an oligonucleotide substrate, we first demonstrated that site-specific DNA cleavage by either yeast- or baculovirus-expressed recombinant human topo I is inhibited by PAR in a dose-dependent manner (Fig. 1A), as previously reported by us for topo I purified from human placenta (24). This inhibition is accompanied by the formation of a PAR-topo I complex (24); degradation of PAR to monomeric ADP-ribose abolished the inhibition (Fig. 1C). In order to investigate whether PAR had any influence on topo I kinase activity, we performed an ASF/SF2 phosphorylation assay in the presence or absence of the polymer. Topo I kinase targets the C-terminal RS domain of ASF/SF2 (29). His-tagged ASF/SF2 expressed in bacteria and purified by metal ion affinity chromatography, was used in this study. The recombinant protein migrated as a doublet in SDS-polyacrylamide gels (Fig. 2C); the shorter form is generated by proteolysis at the C-terminus of the protein, which does not affect the protein’s capacity to be phosphorylated by SR-protein specific kinases (29). Recombinant ASF/SF2 was phosphorylated by both topo I and the SR-protein kinase SRPK1 (Fig. 2A,B). However, when PAR was present in the kinase assay reaction mixture, topo I-catalyzed ASF/SF2 phosphorylation was dramatically reduced (Fig. 2B,E); the extent of inhibition was dependent on PAR concentration (Fig. 2B,E). Thus, PAR appears to function as a negative regulator not only of the DNA cleavage (Fig. 1A) but also of the protein phosphorylation activity of topo I (Fig. 2B,E). Neither PARP-1 nor PARP-2 in their native state affected topo I-catalyzed ASF/SF2 phosphorylation (Fig. 2D). Furthermore, the polymer at several fold higher concentrations than that inhibitory on topo I, had only modest consequences on ASF/SF2 phosphorylation by SRPK1 (Fig. 2A,E), thus pointing at a topo I-specific PAR effect. Noteworthy, while both topo I and SRPK1 phosphorylate serine residues in the RS-domain of ASF/SF2, the reaction catalyzed by topo I has different specificity and kinetics from that of SRPK1 (29,30), implying the involvement of distinct mechanisms.

PAR and ASF/SF2 reciprocally antagonize their topo I inhibitory action. ASF/SF2 is known to inhibit DNA relaxation by topo I by interfering with the DNA cleavage step of the catalytic cycle (25,31-33). In Fig. 1B inhibition of topo I activity was achieved at an approximate topo I:ASF/SF2 molar ratio of 1:10. As both PAR and ASF/SF2 are negative regulators of topo I catalyzed DNA cleavage (Fig. 1), and ASF/SF2 phosphorylation is inhibited by PAR as well (Fig. 2), would PAR and ASF/SF2 together cause a complete silencing of topo I functions? To address this question, we set up an in vitro assay that allows simultaneous detection of topo I-DNA cleavage complex and phosphorylated ASF/SF2. Surprisingly, we observed a full restoration of topo I-catalyzed DNA cleavage while the inhibitory effect of PAR on topo I-dependent ASF/SF2 phosphorylation persisted (Fig. 3A). Reversal of topo I activity on DNA in the presence of both PAR and ASF/SF2 was clearly dependent on PAR concentration (Fig. 3A, right panel) and also occurred in the absence of ATP (Fig. 3B, left panel). It is noteworthy that cleavage complex formation by a N-terminally truncated form of topo I (ΔN-topo I, 70 kDa), which maintained full proficiency to relax DNA but had dramatically reduced protein kinase activity, was also inhibited by either PAR or ASF/SF2 individually (Fig. 3B, right panel). This is in agreement with our previous identification of PAR binding sites outside of the N-terminal domain of topo I (24). Like full length topo I, cleavage complex formation by ΔN-topo I was also restored in the presence of both PAR and ASF/SF2 (Fig. 3B, right panel).

ASF/SF2 is a novel member of the PAR-binding protein family. We next hypothesized that PAR could bind to ASF/SF2 and that the formation of a stable PAR-ASF/SF2 complex might prevent either of the two interaction partners from binding topo I; as a consequence, topo I would be able to express its DNA cleavage activity. PAR binding was assessed using recombinant ASF/SF2, either His-tagged or as a fusion protein with GST. To identify the
specific domain(s) potentially involved in polymer binding, we constructed ASF/SF2 deletion mutants, each comprising only one of the ASF/SF2 functional domains (RRM1/RRM2/RS) (Fig. 4A). PAR binding assays revealed that ASF/SF2 does indeed bind PAR (Fig. 4C,D). We identified the RRM1 and RS domains as potential targets of such interaction, while the RRM2 domain did not appear to possess any PAR binding activity (Fig. 4C,D). Identical results were obtained both when proteins were first separated by SDS-PAGE and then transferred onto nitrocellulose by western blotting (Fig. 4B,D) and when native proteins where immobilized on the membrane by slot-blotting (Fig. 4C), before being probed with radioactive PAR. Importantly, ASF/SF2-PAR binding appeared to be considerably stronger than topo I-PAR interactions: nearly 90% topo I binding to PAR was destroyed by 0.5M NaCl; in contrast, most ASF/SF2-PAR complexes resisted high salt treatment (Fig. 5A).

Next, the inhibitory effect of ASF/SF2 on DNA cleavage by a constant amount of topo I was titrated against increasing concentrations of PAR. We found that restoration of topo I DNA cleavage activity was strictly dependent on the relative amounts of the two negative effectors (Fig. 5B): 10 pmol polymeric ADP-ribose were sufficient to fully remove inhibition caused by 2.8 pmol ASF/SF2; at higher PAR concentrations however, cleavage complex formation decreased again, probably as a consequence of topo I targeting by PAR molecules that exceeded ASF/SF2 binding capacity.

ASF/SF2-, but not histone- phosphorylation by HeLa nuclear extracts is inhibited by PAR. Is PAR able to modulate ASF/SF2 phosphorylation in a complex protein environment such as a nuclear extract? Fig. 6A demonstrate that PAR inhibits ASF/SF2 phosphorylation activity in HeLa nuclear extracts in a dose-dependent manner. Notably, preincubation with DNA and CPT, but not with CPT alone, strongly reduced ASF/SF2 phosphorylation (Fig. 6B). Such a treatment has been demonstrated to cause specific inhibition of topo I kinase activity in HeLa nuclear extracts as a consequence of drug-induced topo I trapping in an inactive cleavage complex (6).

In addition, the inhibitory action of PAR appeared to be selectively targeted to ASF/SF2 kinase(s); histone H1 and H2B phosphorylation was not inhibited by PAR over the same dose range (Fig. 6C).

Phosphorylation of endogenous nuclear extract proteins also appeared to be unresponsive to PAR (Fig. 6A and C, upmost part of the gels).

DISCUSSION

Human DNA topoisomerase I plays an important role in the regulation of gene expression by controlling DNA supercoiling on one side, and pre-mRNA splicing, on the other. In fact, in addition to relaxing supercoiled DNA, topo I exhibits a kinase activity targeted at the Serine-Arginine rich family of splicing factors (SR-proteins). How these functions are regulated is still unknown. Significantly, pharmacological inhibition of either of the two enzyme activities alters gene expression and eventually leads to cell death (3-5,17).

Here we show that PAR is a fine regulator of topo I functions (cf. Fig. 7). Both PAR and the splicing factor ASF/SF2 bind topo I and inhibit topo I-catalyzed DNA cleavage (24,25,31-33). Likewise, in the absence of ATP, PAR and ASF/SF2 each caused a dose-dependent silencing of topo I activity on DNA (Fig. 1). However, when these negative effectors were present simultaneously, the outcome was different and entirely unexpected: PAR and ASF/SF2 neutralized each other’s inhibitory actions and allowed restoration of the enzyme’s DNA cleavage activity (Fig. 3B). Thus PAR enabled topo I to direct its activity onto DNA under conditions that would otherwise be strongly inhibitory. In a more complex setting, with DNA, ATP and ASF/SF2 being incubated simultaneously with topo I, we observed that the enzyme phosphotransferase activity was by far favoured over the DNA cleavage activity; under such conditions topo I appeared in fact to be a more efficient kinase than a topoisomerase (Fig. 3A); this is in agreement with observations reported by others (31). In addition, we found that PAR is able to restore topo I activity on DNA while at the same time reducing (but not abolishing) topo I-dependent ASF/SF2 phosphorylation; the extent at which distinct topo I functions are either reactivated or inhibited appeared to be dependent on the amount of PAR (Fig. 3A, right panel). A mechanistic explanation of such an effect is offered by the finding that ASF/SF2 is a novel PAR binding partner, with the RRM1 and RS domains as specific PAR interaction targets (Fig.
4). Conceivably the formation of a PAR-ASF/SF2 high affinity complex leaves topo I available for directing its action on DNA. Indeed, PAR-ASF/SF2 titration experiments (Fig. 3 and 5B) as well as the finding that PAR binds to ASF/SF2 more tightly than to topo I (Fig. 5A), are consistent with such a model. The fact that SRPK1, another SR-protein specific kinase, is only partially sensitive to PAR (Fig. 2) further emphasizes the specificity of PAR effects on topo I activities. Thus, PAR appears to be the first known physiological modulator of topo I kinase activity. Moreover, we found that ASF/SF2- but not histone phosphorylation by HeLa nuclear extracts is inhibited by PAR (Fig. 6), suggesting that PAR might play a role as a regulator of ASF/SF2 phosphorylation status in the nucleus as well. Noteworthy, endogenous topo I could be identified as the main ASF/SF2 kinase in the extract: in fact, enzyme trapping in a ternary complex with CPT and cleaved DNA abolished most of the ASF/SF2 phosphorylating activity (Fig. 6B). In future studies it will be of interest to determine whether other members of the SR-protein family, that are also phosphorylated by topo I (6), may become targets of a PAR-reliant control mechanism.

How might regulation of topo I functions by PAR affect biological processes? We propose a model by which PAR, through modulation of topo I substrate selection (either DNA or ASF/SF2), may participate in the coordination of DNA transcription and RNA splicing. Indeed it is now well established that RNA splicing and transcription are spatially and functionally coupled (34,35). This integration ensures that pre-mRNA processing is efficient and accurate and at the same time may prevent formation of potentially lethal DNA-RNA hybrids involving template DNA and nascent mRNA precursors (10). By virtue of its dual activity, involving control of DNA topology on one side and phosphorylation of splicing factors on the other, topo I may take part in transcription-splicing coupling mechanisms (31). Besides, several lines of evidence support a likely functional link between topo I and PARP-1 in the regulation of gene expression. In fact, both topo I and PARP-1 localize to active transcription sites (1,2,36,37); furthermore, the two enzymes may form a complex resulting in a several fold enhancement of topo I DNA relaxation activity and/or loss of interaction with other protein partners (37-39). Additionally, ASF/SF2, topo I and PARP-1 share a high affinity for the phosphorylated carboxy-terminal domain of RNA polymerase II and can be associated with the elongating polymerase in vivo (40). In the light of such evidences, we propose that topo I targeting by PAR may play a role during transcription elongation, when topo I activity is required both to relieve torsional stress generated by RNA polymerase II translocation along the DNA template (41), and to phosphorylate ASF/SF2 engaged in co-transcriptional splicing events (16,17). The nick introduced onto the DNA template by topo I itself might constitute the activating signal for PARP-1, in analogy with the mechanism underlying PARP-1 and DNA topoisomerase II β cooperation at the level of transcription initiation (42). Alternatively, PAR synthesis might be stimulated by PARP-1 binding to altered DNA structures (43) that are formed during transcription. Newly synthesised PAR might then exert its regulatory role at two levels: 1) by attenuating (but not abolishing) topo I kinase activity that would be otherwise greatly favoured by the high local concentration of ASF/SF2, and 2) by neutralizing the strong ASF/SF2 inhibitory effect on topo I-catalyzed DNA relaxation. Thus, an interplay between the levels of PAR and ASF/SF2 at transcription sites may control topo I activities and adds a further layer of regulation of gene expression.

Significantly, the amounts of PAR required to regulate topo I and induce the enzyme’s functional switch in vitro (5-20 pmol, i.e. 0.3-1.3 μM, calculated as ADP-ribose moieties), are compatible with PAR cellular levels (0.11-1.3 μM under physiologic conditions - up to 35 μM following DNA damage) (44 and references therein,45)

Lastly, several lines of evidence hint at a likely more direct involvement of the poly(ADP-ribosyl)ation system in pre-mRNA processing: i) PAR binds to components of the RNA splicing machinery, i.e. the splicing factor ASF/SF2 (this study, Fig. 4) and several hnRNPs (46); ii) both PARP and PARG activities have been found in cytoplasmic ribonucleoprotein particles (47,48); iii) PARP-1 has been shown to possess RNA-binding ability (49). The observation that ASF/SF2 phosphorylation – but not histone phosphorylation - in HeLa nuclear extracts is sensitive to PAR (Fig. 6) is also consistent with such a scenario. Moreover, the ASF/SF2 domains targeted by PAR, i.e. the RRM1 and RS domains, are crucial for splicing functions, suggesting that PAR effects may extend beyond regulation of ASF/SF2 phosphorylation. Notably, the RS domain, which is present in several splicing factors, when tethered to a pre-
mRNA is sufficient alone to promote pre-spicosome assembly and to support splicing (50). A speculation along this line is also supported by our results of an *in silico* study on published expression profiling data from wild type and *parp-1* knockout cells and animal tissues (51-55). We found that within a group of genes involved in the response to cytotoxic stimuli, the incidence of alternative splicing increases specifically in the subset of *parp-1* dependent genes (Tab. I). The fraction of alternatively spliced genes in this subset was 67.7-72.2% compared to 53-59% in the group of stress-regulated genes as a whole. Noteworthy, of the 18 genes that were reported to be differentially involved in the TNF-α, response (54), 10 were not responsive either in wild type or knockout cells (possibly because of altered signaling), while 8 genes changed their expression levels in both cell types, but at different extents; the incidence of alternative splicing in this subgroup of genes was found to be 87.5% and reached 100% when considering only those genes (5 out of 8) that were expressed at lower levels in cells lacking a functional *parp-1*; moreover, all these genes contained ASF/SF2 binding sites (data not shown). It should be emphasized that the number of published data that could be included in this study does not allow any conclusive statement yet. Nevertheless, our observations suggest a possible link between PAR/PARP-1 and alternative splicing which warrants further validation and investigations.

In conclusion, PAR may function as a molecular switch of topo I DNA cleavage/SR-protein kinase activities. We speculate that PAR targeting of topo I and/or SR-protein functions may play a role as a novel regulatory mechanism of gene expression. Such a mechanism might involve a specific set of genes and entail regulation of topo I-dependent phosphorylation of SR-proteins and/or modulation of SR-proteins’ interaction with splicing coactivators/corepressors. Important clues may come from the identification of target genes, an achievement that should be greatly accelerated as more differential expression profiling data become available.
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**FOOTNOTES**

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The abbreviations used are: ASF/SF2, alternative splicing factor/splicing factor 2; CPT, camptothecin; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase); PARP, poly(ADP-ribose) polymerase; SRPK1, SR-protein specific kinase 1; topo I, DNA topoisomerase I.
FIGURE LEGENDS

Fig. 1. Both PAR and ASF/SF2 independently inhibit DNA cleavage by human recombinant topo I. Human recombinant topo I expressed either in yeast or, as a His-tagged protein, in a baculovirus system was incubated with a 5'-[\(^{32}\)P]ds-oligonucleotide containing a single topo I cleavage site in the absence or presence of either affinity purified PAR (A and C) or ASF/SF2 (B), as described under Materials and Methods. Panel C shows restoration of topo I DNA cleavage activity after PAR enzymatic digestion by PAR glycohydrolase (PARG). Topo I-DNA cleavage complexes were visualized by autoradiography following SDS-PAGE.

Fig. 2. PAR inhibits ASF/SF2 phosphorylation. ASF/SF2 phosphorylation by either SRPK1 (0.05 pmol; A) or topo I (0.2 pmol; B) was assayed in the absence or presence of increasing amounts of PAR; alternatively, 0.18 pmol of native PARP-1 or 0.3 pmol of PARP-2 were added together with topo I and ASF/SF2 into the kinase assay mixture (D). Phosphorylated ASF/SF2 was visualized by autoradiography following SDS-PAGE (A,B,D). Duplicate gels were stained with Coomassie Blue for protein visualization (C). Autoradiographic bands were quantified by scanning densitometry and data were expressed as percent of enzyme activity in the absence of PAR (E); data from at least 3 independent experiments (mean ± SD) are shown.

M: molecular mass markers; size of protein markers (kDa) are indicated by numbers on the left in panel C

Fig. 3. PAR removes ASF/SF2 inhibition on topo I DNA cleavage activity while modulating topo I protein phosphotransferase activity. Double DNA cleavage and ASF/SF2 phosphorylation assays (A) were performed as described in Materials and Methods, in the presence of both [\(^{32}\)P]-ds-oligonucleotide and [\(^{32}\)P]-ATP: simultaneous detection of cleavage complexes and phosphorylated ASF/SF2 was achieved by autoradiography following electrophoretic separation on polyacrylamide gradient gel (left). In similar assays, the effects of fixed amounts of ASF/SF2 (2.8 pmol) on topo I activities were titrated against increasing amounts of PAR and quantified by densitometric scanning of autoradiographic bands. Data are expressed as percent of topo I activity in the absence of PAR (line graph on the right: mean ± SD of at least three independent experiments).

In the absence of ATP (B), DNA cleavage by either full length recombinant human topo I (gel on the left) or N-terminally truncated topo I (\(\Delta\)N-topo I) from human placenta (gel on the right), was carried on under the conditions described in the legends to Fig. 1. Full length topo I-DNA complex (faint band in the first lane of the gel on the right) was loaded on this gel as a marker (M) to allow for a direct comparison with the migration of \(\Delta\)N-topo I-DNA complex.

*P-ASF/SF2: phosphorylated ASF/SF2.

Fig. 4. ASF/SF2 is a PAR binding protein. Bacterially expressed recombinant ASF/SF2 or protein domains (A) were immobilized onto nitrocellulose by either western- (B,D) or slot-blotting (C). Duplicate samples were either stained for protein visualization or incubated with [\(^{32}\)P]-PAR and autoradiographed for PAR-protein complex determinations. 1.5 µg of each protein was loaded on SDS-polyacrylamide gels; the amounts of slot-blot native proteins ranged from 2.5 pmol (recombinant proteins 2-5) to 10 pmol (GST); the amounts of His-ASF/SF2 (1) used in the slot blot PAR binding assay were 2.5 (1') and 5 pmol (1'').

Fig. 5. PAR binds to ASF/SF2 with higher affinity than to topo I. A): effect of high salt treatment on the stability of PAR-protein complexes. Recombinant proteins (5 pmol) were immobilized on nitrocellulose by slot blotting and either Gold stained (inset: a) or incubated with [\(^{32}\)P]-PAR, followed by washes with either TBST or 0.5 M NaCl-TBST (inset: b and c, respectively). PAR-protein complexes were visualized by autoradiography (inset: b,c) and band intensities were quantified by scanning densitometry. Data are expressed as percent of controls (no salt treatment) and represent mean values ± SD of at least three independent experiments.

B): competition binding functional assay. Topo I (0.2 pmol) was incubated with 5'-[\(^{32}\)P]ds-oligonucleotide substrate and ASF/SF2 in the absence or presence of increasing amounts of PAR. Topo I-DNA cleavage complexes were visualized by autoradiography following SDS-PAGE and
quantified by scanning densitometry. Data shown represent the mean ± SD of at least three independent experiments.

Fig. 6. ASF/SF2-, but not histone phosphorylation by HeLa nuclear kinase(s) is inhibited by PAR. Protein phosphorylation assays were carried on in the absence or presence of increasing amounts of PAR, using 1 μg of HeLa nuclear protein and 7 pmol of either ASF/SF2 (A,B) or histones, i.e. H1 (9 pmol) or H2B (14.5 pmol) (C). Lane 1 of the gel in A shows endogenous extract protein phosphorylation: no exogenous kinase substrate was added to the reaction mixture. In the experiment shown in B, ASF/SF2 phosphorylation was assayed in the presence of increasing amounts of CPT +/- plasmidic dsDNA. Phosphorylated proteins were visualized by autoradiography following incubation with [γ-32P]-ATP and separation by SDS-PAGE.

Fig. 7. Regulation of topo I functions by PAR. The model illustrates PAR effects on distinct topo I activities and the likely mechanism underlying PAR-induced topo I functional switch. PAR interacts both with topo I and ASF/SF2 (double pointed arrows), with the latter forming more stable complexes (thicker double pointed arrow). In the absence of ASF/SF2, PAR targeting causes topo I to lose its DNA cleavage activity (A). ASF/SF2 is also a binding partner for topo I and a negative regulator of topo I catalyzed DNA cleavage; moreover, in the presence of ATP, ASF/SF2 reactivates a latent topo I kinase activity and becomes itself phosphorylated (B). When PAR, topo I and ASF/SF2 are present simultaneously, the two proteins compete for PAR binding: the formation of a PAR-ASF/SF2 high affinity complex leaves topo I free to direct its action on DNA (C). In such a case, PAR acts as a modulator of the relative expression levels of topo I activities; in fact, whether topo I functions as a topoisomerase or as a kinase depends on PAR amounts: at low PAR concentrations, both DNA cleavage and protein phosphorylation can occur (C, left); DNA cleavage activity is fully restored (at the detriment of the kinase activity) at higher PAR concentrations that titrate off ASF/SF2 from its interaction with topo I (C, right).
Table 1 – Incidence of alternative splicing among genes that are differentially expressed in cells or tissues lacking parp-1 and in their wild type counterparts, under physiological conditions or after cytotoxic stimuli

| Cell type/tissue       | treatment | differentially expressed genes | alternatively spliced genes (%) | Data source (Ref.) |
|------------------------|-----------|--------------------------------|--------------------------------|-------------------|
| **parp-1−/− versus wild type** |           |                                |                                |                   |
| Primary MEF            | none      | 58                             | 30 (51.7)                      | Simbulan-Rosenthal et al (51) |
| MES                    | none      | 291                            | 164 (56.3)                     | Dechenes et al (52) |
| MES                    | none      | 86                             | 46 (53.4)                      | Ogino et al (53)   |
| Mouse liver            | none      | 26                             | 15 (57.6)                      | Ogino et al (53)   |
| **Wild type treated versus wild type untreated** |         |                                |                                |                   |
| MHEC                   | TNF-α     | 43*                            | 23 (53.5%)                     | Carrillo et al (54) |
| **parp-1−/− treated versus parp-1−/− untreated** |         |                                |                                |                   |
| MHEC                   | TNF-α     | 39*                            | 23 (58.9%)                     | Carrillo et al (54) |
| **parp-1−/− treated versus wild type treated** |         |                                |                                |                   |
| MHEC                   | TNF-α     | 18*                            | 14 (72.2)                      | Carrillo et al (54) |
| Mouse heart            | ischemia-reperfusion | 31*                          | 21 (67.7)                      | Zingarelli et al (55) |

MEF: mouse embryonic fibroblasts; MES: mouse embryonic stem cells; MHEC: mouse heart endothelial cells.

*All these genes were similarly expressed in both parp-1−/− and wild type untreated cells/tissues
Fig. 1
Fig. 2
Fig. 3
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Fig. 5
Fig. 6
Fig. 7