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Co-Localization of Poly(ADPR)Polymerase 1 (PARP-1) Poly(ADPR)Polymerase 2 (PARP-2) and Related Proteins in Rat Testis Nuclear Matrix Defined by Chemical Cross-Linking

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Abstract Poly(ADPR)polymerase 1 and 2 (PARP-1, PARP-2) are nuclear enzymes which function is based on specific interactions with DNA and nuclear proteins. PARPs targets include proteins involved in DNA replication, repair, and transcription and their function can be modulated either by protein–protein interaction with native PARP-1 and by non-covalent interaction with poly(ADP-ribose) (pADPR) linked to the auto-modified PARP-1. Moreover, the association of pADPR and PARP-1 with the nuclear matrix (NM) has been reported, based on the poly(ADP-ribosyl)ation of nuclear matrix proteins (NMPs). In the present article, by the use of DNA and protein cross-linking reactions, by *cis*-diamminedichloroplatinum II (cDDP) and sodium tetrathionate (NaTT) respectively, we present more evidences about the association of PARP-1, PARP-2, and PARPs related proteins with the NM. Our findings confirmed that NM could be seen as a fraction greatly enriched in transcription factors (i.e., C/EBP-β) and enzymes (DNA Topo II, DNA PK) that co-localize with PARP-1 and -2 at the matrix associated regions (MARs) of chromatin. Moreover, pADPR contributes to PARP-1 localization at the NM, showing that PARP(s) activity co-operates to the functions of this nuclear fraction. J. Cell. Biochem. 94: 58–66, 2005.

Key words: PARP-1; PARP-2; DNA–protein cross-linking; protein–protein cross-linking; nuclear matrix association

The poly(ADPR)polymerase 1 (PARP-1) is a nuclear enzyme that catalyzes the attachment of ADP-ribose units from NAD^+ substrate to itself and to a variety of nuclear proteins (histones nuclear enzymes, transcription factors, etc.) thus regulating their functions [Rolli et al., 2000]. The polymer poly(ADP-ribose) (pADPR) may extend to 200 residues and its level is 10–500 fold increased in response to DNA damage [Bürkle, 2001]. Although the main role of PARP-1 is related to the DNA repair and/or apoptosis induction [Scovassi and Poirier, 1999; Bürkle et al., 2000; Bürkle, 2001], more recent studies indicated that a background PARP-1 activity in normal cells can regulate gene expression by modulating chromatin structure and transcriptional activity [Ziegler and Oei, 1999; Kraus and Lis, 2003]. Indeed, PARP-1 function is based on specific interactions with DNA and nuclear proteins.

PARP-1 is a 116 kDa protein with a modular structure [Rolli et al., 2000]. The N-terminal DNA binding domain contains two zinc finger motifs and a bipartite nuclear localization signal. The central region of the protein bears the auto-modification domain and a BRCT motif responsible for specific protein–protein interaction in several pairs of DNA repair proteins [Callebaut and Mornon, 1997]. The C-terminal domain contains the catalytic NAD^+ binding domain. This latter is conserved in all the members of the PARP family identified in the last years, the PARP-2 [Amé et al., 1999], the PARP-3 [Johansson, 1999], the sPARP [Sallman et al., 2000], the tankyrase localized at human telomers [Smith et al., 1998], the vPARP

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component of the Vault ribonucleoprotein complex [Kickhoefer et al., 1999], and the TiPARP [Ma et al., 2001]. Among them PARP-2, a 62 kDa PARP isoform, contains a small highly basic N-terminal DNA binding domain and a central dimerization and auto-modification domain. Moreover, PARP-2 associates with PARP-1 and XRCC-1 for an efficient base excision repair [Schreiber et al., 2002].

The counterpart enzyme of PARPs is the poly(ADPR)glycohydrolase (PARG) which is recruited into the nucleus to ensure rapid turnover of the polymer and to restore enzymatic activity of auto-modified PARP-1 [Davidovic et al., 2001].

PARP-1 target proteins include, beside itself, proteins involved in DNA replication and repair (DNA polymerases, DNA ligase, DNA topoisomerases, DNA PK) in transcription (Oct-1, YY-1, NFkB) and cell cycle control (p53) [Ziegler and Oei, 1999; Bürkle, 2001; Kraus and Lis, 2003]. These proteins can be modulated in their functions either by protein–protein interaction with native PARP-1 and by non-covalent interaction with pADPR linked to the auto-modified PARP-1. Among others, histones, heterogenous nuclear ribonucleoproteins, lamins, p53 and p21, contain pADPR binding motifs consisting of alternating hydrophobic and basic residues [Pleschke et al., 2000].

Although PARP-1 is activated only by DNA breaks, in vitro PARP-1 binds to other DNA structure such as cruciform or curved DNA [Soldatenkov et al., 2002] and this property has been related to its function at the nuclear matrix (NM) [Bürkle et al., 2000]. The association of pADPR and PARP-1 with the NM has been reported [Cardenas-Corona et al., 1987; Alvarez-Gonzalez and Ringer, 1988; Quesada et al., 2000] based also on the poly (ADP-ribosyl)ation of nuclear matrix proteins (NMPs) (lamins, DNA topoisomerase II) [Bürkle et al., 2000; Gagnè et al., 2003].

The NM is a sub-nuclear structure resistant to nuclease digestion and high salt extraction composed by the nuclear lamina, nucleoli, and a fibro-granular internal matrix [Nickerson, 2001; Jackson, 2003]. The NMPs are characterized by putative zinc fingers motifs, which suggest similarity with DNA binding proteins [Nakayasu and Berezney, 1991]. The NM has a role in the organization and function of DNA. The chromatin fiber is organized into loop domains by the association of DNA sequences, called matrix associated regions (MARs) located at the basis of the loops, with NM proteins [Heng et al., 2004]. MARs flank transcribing chromatin and contain NM-bound transcription factors and histone modifying enzymes (C/EBP-β, HDAC-1 and -2, DNA PK, PARP-1) [Hendzel et al., 1991; Desnoyers et al., 1996; Galande and Kohwi-Shigematsu, 1999; Uskoković et al., 2002]. On the basis of all these notions, NMPs have been involved in the control and coordination of gene expression.

We already published that the rat testis NM contains an active poly(ADP-ribosyl)ation system characterized by either the auto-modification of PARP-1 and the hetero-modification of core histones tightly bound to the NM [Quesada et al., 2000]. In the present article, by the use of DNA and protein cross-linking reactions we present more evidences about the association of PARP-1 plus PARP-2 and PARP(s) target proteins to the NM. Our findings suggest that the form of PARP-1 tightly associated to the NM co-localizes with several potential partners in this sub-nuclear fraction.

MATERIALS AND METHODS

Materials

[^{14}C]NAD$^+$ nicotinamide (U^{14}C adenine dinucleotide ammonium salt) (250 mCi/mmol) was supplied by Amersham Italia S.r.l., Italy. The cocktail of protease inhibitors was from Roche-Biochemical (Basel, Switzerland). Anti-PARP-1 rabbit polyclonal antibody (H-250), anti-C/EBP-β rabbit polyclonal antibody (C-19), anti-DNA topoisomerase II goat-polyclonal (N16), anti-lamin B rabbit polyclonal antibody (C-20), anti-Ku70 rabbit polyclonal antibody (H-30P), anti-XRCC-1 rabbit polyclonal antibody (H-300), anti-RAD-51 rabbit polyclonal antibody (H-92), and goat anti-rabbit IgG HRP-conjugate or goat anti-mouse IgG HRP-conjugate were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PARP-2 rabbit polyclonal antibody and anti-pADPR mouse monoclonal antibody (H10) were from Alexis Biochemicals (Vinci, Italy). All other chemicals were of the highest quality commercially available.

Induction of Protein–Protein Cross-Linking by Sodium Tetrathionate (NaTT)

Rat testis nuclei isolated by homogenization and differential centrifugation [Quesada et al.,

2000] were resuspended (2 mg/ml of DNA) in 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 50 mM NaCl, 250 mM sucrose, 0.5 mM EGTA (Buffer A), and a 1:25 dilution of a cocktail of protease inhibitors. Cross-linking reaction was induced by 1 mM NaTT in Buffer A for 30 min at 4°C. After centrifugation 2,000*g* 15 min at 4°C the nuclei were used for NM isolation.

Induction of DNA–Protein Cross-Linking by *cis*-Diamminedichloroplatinum II (cDDP)

The DNA–protein cross-linking was performed on isolated nuclei (2 mg/ml of DNA). The nuclei were resuspended in the Hanks solution: 137 mM NaCl, 1. 8 mM $CaCl_2$, 1.6 mM $MgSO_4$, 2.7 mM KCl, 0.73 mM KH_2PO_4, 15 mM Na bicarbonate, 2 g/L glucose, 1 mM PMSF. cDDP was added at a final concentration of 1 mM and the suspension incubated for 2 h at 37°C. After centrifugation at 2,000*g* for 5 min at 4°C and several washes in Hanks solution the nuclei were used for NM isolation.

The reversion of the cDDP cross-linking of nuclei and NM was made by incubation with 1 M thiourea, 2 M NaCl, 2 M guanidine–HCl in 200 mM KH_2PO_4 pH 7.5 for 2 h at 4°C.

Isolation of NM

For NM isolation the nuclei were subjected to digestion with 250 μg/ml DNase I in digestion buffer (300 mM sucrose containing 10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 1 mM PMSF) and consecutive extractions/centrifugations: twice with a high salt buffer (250 mM $(NH_4)_2SO_4$, 0.2 mM $MgSO_4$, 1 mM PMSF in 10 mM Tris-HCl pH 7.4), once with low salt buffer (0.2 mM $MgCl_2$, 1 mM PMSF in 10 mM Tris-HCl pH 7.4). After centrifugation at 2,000*g* 15 min at 4°C, the NM was recovered as a pellet and re-suspended in 250 mM sucrose, 0.2 mM $MgCl_2$, 1 mM PMS, 10 mM Tris-HCl pH 7.4.

Isolation of internal NM was made according with Stuurman et al. [1990] by extraction with 2 M NaCl, 20 mM DTT, 0.2 mM $MgSO_4$, 1 mM PMSF in 10 mM Tris-HCl pH 7.4, for 20 min on ice, preceded by overnight digestion at 4°C with 250 μg/ml DNase I and 250 μg/ml RNase A in digestion buffer. After centrifugation at 10,000*g* for 20 min at 4°C the internal NM was recovered in the supernatant.

Solubilization of NMP was carried out in 8 M urea, 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM PMSF, 1% β-MeETOH, in 20 mM MES pH 6.6 for 45 min at 30°C. After centrifugation at 10,000*g* for 15 min at room temperature the supernatant was dialyzed over night against 1,000 volumes of the same buffer without urea.

Poly(ADP-Ribosyl)ation Reaction

Intact nuclei resuspended (50×10^6 nuclei/ml) in 10 mM Tris-HCl pH 8, 14 mM β-MeETOH, 10 mM $MgCl_2$, 60 mM NaCl, 1 mM PMSF, containing 250 mM sucrose were incubated with 200 μM [^{14}C]NAD^+ (10,000 cpm/nmole). After 20 min incubation at room temperature, the reaction was stopped by the addition of ice-cold tricloroacetic 40% (v/v) and the radioactivity associated to the acid-insoluble materials counted on a Beckman LS 8100 liquid scintillation spectrometer.

The nanomoles of poly(ADP-ribose) were calculated on the basis of the specific radioactivity of [^{14}C]NAD^+ of 10,000 cpm/nmole. The data are means of three experiments done in duplicate ±SE.

Western Blotting Experiment

Nuclei and NM (80–100 μg proteins) were separated by SDS–PAGE (5–15% gradient gels) and transferred onto a polyvinylidene-fluoride (PVDF) membrane using an electroblotting apparatus. The membrane was blocked with 5% non-fat milk in 20 mM Tris-HCl pH 8, 150 mM NaCl and 0.5% Tween 20 (TBST) overnight and subsequently incubated with anti-PARP-1 antibody diluted 1:200, anti-PARP-2 diluted 1:5,000, anti-lamin B diluted 1:2,000, anti-C/EBP-β diluted 1:500, anti-DNA topoisomerase II diluted 1:200, anti-Ku70 diluted 1:200, anti-XRCC-1 diluted 1:1,000, anti-RAD-51 diluted 1:1,000, and anti-pADPR diluted 1:250 in 3% non-fat milk in TBST for 2 h at room temperature. Unbound antibody was removed by washing 3× with TBST and the membrane was incubated with goat anti-rabbit IgG HRP-conjugate diluted 1:4,000, or goat anti-mouse IgG HRP-conjugate diluted 1:2,000, in 3% non-fat milk in TBST for 1.5 h at room temperature. The membrane was washed 3× in TBST and enzyme activity detected using the Luminol reagent (Santa Cruz). Control blots omitted the first layer antibody. The density of the immunoreactive bands was quantified using the Immuno-Star Chemioluminescent detection system (BioRad, Milan, Italy).

Protein and DNA Assay

Protein concentration was determined using the Bradford protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

DNA concentration was determined by the diphenylamine method using calf thymus DNA as a standard.

RESULTS

Protein–Protein Cross-Linking Induced by NaTT in Rat Testis Nuclei and NM

In this study, we used cross-linking with NaTT to examine the relationship between PARPs and other NMPs. Figure 1 shows that the induction of disulfide bonds by NaTT determined an electrophoretic mobility shift of several nuclear proteins analyzed by Western blotting after SDS–PAGE. The immunoreactive band of PARP-1 in correspondence to 116 kDa MW disappeared when β-MeEtOH was omitted in the sample buffer: the broad band in the correspondence of a 200–250 kDa range of MW is indicative of protein–protein complex(es). Moreover, the PARP-1 immunoreactive band was present again in the sample run in the presence of the reducing agent. The same happened to PARP-2 and XRCC-1 two proteins that is known to physically interact with PARP-1 [Schreiber et al., 2002]. Figure 1 also shows the same behavior for C/EBP-β a protein that was never related to PARP enzymes but it is known to be associated with the NM [Uskoković et al., 2002]. Vice versa the protein RAD-51 a component of the nuclear foci involved in homologous recombination [Haaf et al., 1995] shows the same electrophoretic mobility in the samples treated or not with NaTT as an indication that not all the nuclear proteins undergo the consistent cross-linking.

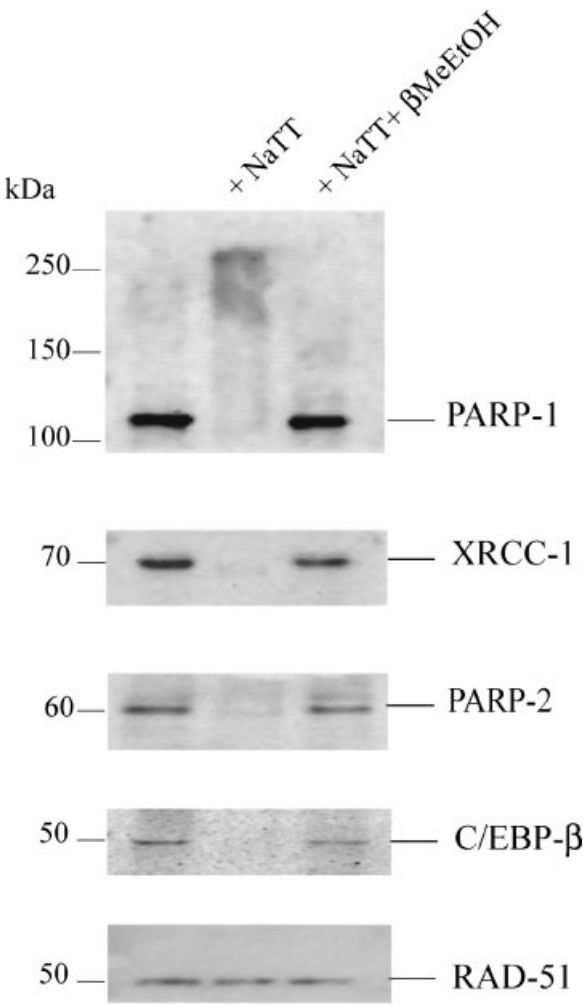

Fig. 1. Western blotting analysis of protein extracts (80 μg) from rat testis nuclei cross-linked or not with 1 mM sodium tetrathionate (NaTT) after 5–15% SDS–PAGE in the presence or absence of β-MeEtOH. Immuno-staining with polyclonal antibodies anti poly(ADPR)polymerase 1 (PARP-1) (diluted 1:2,000), PARP-2 (diluted 1:5,000), XRCC-1 (diluted 1:1,000), C/EBP-β (diluted 1:500), and RAD-51 (diluted 1:1,000).

Indeed, it has been reported that the association of proteins with the NM can be assessed by their tendency to crosslink when treated with agents like NaTT [Kaufmann and Shaper, 1984; Desnoyers et al., 1996]. We then analyzed the presence of PARPs and other PARP related proteins in the NM after NaTT cross-linking reaction. Moreover, the NM was isolated by extraction with ammonium sulfate, an agent that largely preserves the nuclear morphology. Figure 2 shows the analysis by Western blotting of several proteins present in the NM isolated from nuclei incubated or not with NaTT. The SDS–PAGE pattern is shown as a control of a comparable loading onto the gel. Immunoreactive bands of different density were seen for proteins like PARP-1 and C/EBP-β. Densitometric analysis indicated that NaTT treatment increased the amount of such proteins in the NM for a comparable amount of lamin B. Moreover, we determined the association with the NM of PARP-2 which light immunoreactive band was not intensified by the NaTT treatment. Densitometric analysis indicated 70–80% of the PARP-2 nuclear amount in both the samples of NM.

Next, we tried to better determine the nuclear localization of PARP-1 by isolating the internal

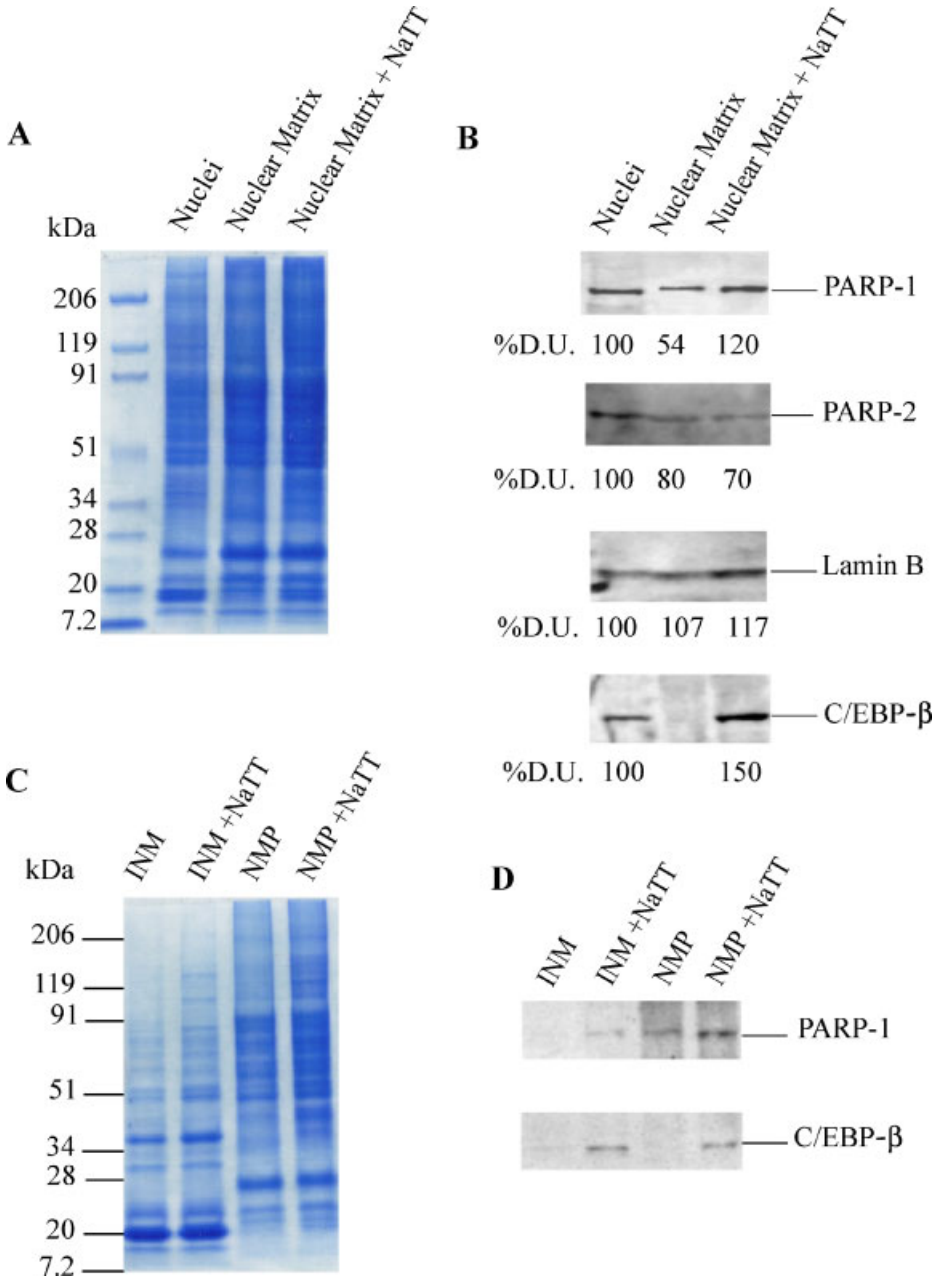

Fig. 2. Western blotting analysis of protein extracts (100 μg) of rat testis nuclear matrix (NM), internal nuclear matrix (INM), nuclear matrix protein (NMP) from nuclei treated or not with NaTT after 5–15% SDS–PAGE in the presence of β-MeEtOH. **A**, **C**: Coomassie-staining of the SDS-gel; (**B**, **D**) immuno-staining with polyclonal antibodies anti-PARP-1 (diluted 1:2,000), PARP-2 (diluted 1:5,000), lamin B (diluted 1:2,000), and C/EBP-β (diluted 1:500). D.U. densitometric units. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nuclear matrix (INM) according to Stuurman et al. [1990] preceded by a DNAse/RNAse digestion, and by solubilization with 8 M urea of NMPs [Davie et al., 1998]. Figure 2C shows the SDS–PAGE proteins pattern that was enriched in components with 100–200 kDa MW in samples from NaTT treated nuclei. Moreover, by Western blotting (Fig. 2D), we found that the amount of PARP-1 was increased in both the NM fractions. Again, NaTT cross-linking allowed the localization of C/EBP-β in these fractions.

DNA–Protein Cross-Linking in Rat Testis Nuclei and NM

To provide evidences of a specific association of PARPs and related proteins with DNA we

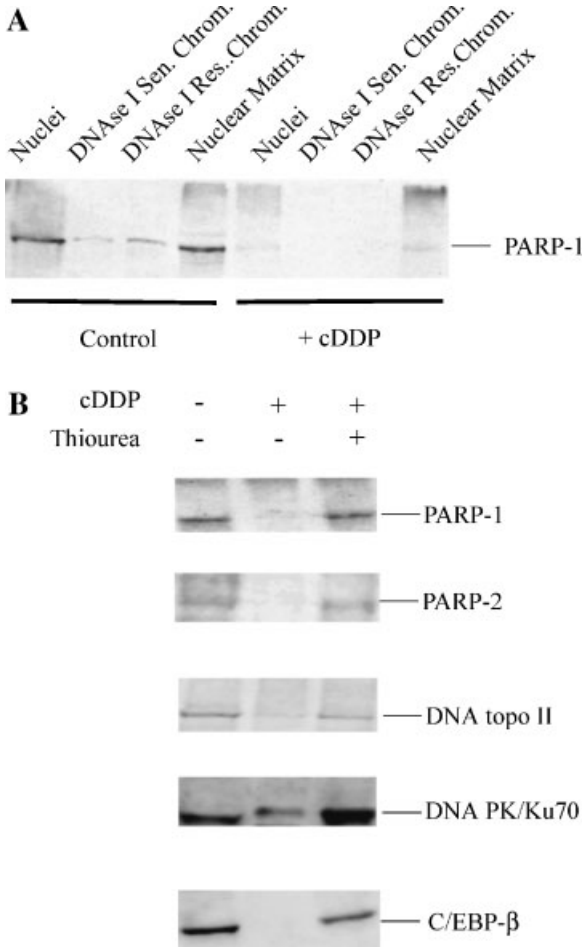

Fig. 3. Western blotting analysis of protein extracts (100 μg) of nuclear fractions from rat testis nuclei cross-linked or not with 1 mM *cis*-diamminedichloroplatinum II (cDDP) after 5–15% SDS–PAGE, treated or not with 1 M thiourea (see ''Materials and Methods''). **A**: Immuno-staining of nuclei, DNase I sensitive and DNase I resistant chromatin, NM, with polyclonal antibody anti-PARP-1 (diluted 1:2,000). **B**: Immuno-staining of NM after cDDP cross-linking reaction and reversion with thiourea with polyclonal antibody anti-PARP-1 (diluted 1:2,000), PARP-2 (diluted 1:5,000), DNA topoisomerase II (diluted 1:200), DNA PK/ Ku70 (diluted 1:200), and C/EBP-β (diluted 1:500).

incubated nuclei with cDDP which is known to preferentially cross-link NMPs to MAR DNA [Mattia et al., 1998]. Figure 3A shows that the immunoreactive band specific for PARP-1, evident in nuclear fractions from control nuclei, was absent in the same fractions isolated from nuclei after cDDP cross-linking reaction. Indeed, in the NM isolated from nuclei treated with cDDP, the rather intense PARP-1 immunoreactive band present in the control sample almost disappeared: a broad band at the top of the PDVF membrane was indicative of a DNA–protein complex that cannot enter the polyacrylamide gel.

To ascertain that this behavior was due to the cross-linking of PARP-1 to matrix associated chromatin regions (MARs), we set-up the conditions for the reversion of the cDDP cross-linking by the use of thiourea (see "Materials and Methods") and we analyzed by Western blotting NM fractions treated or not with such an agent. Figure 3B shows a PARP-1 immunoreactive band of comparable intensity in reversed (thiourea treated) and control sample. Moreover, the same analysis done with an anti-PARP-2 antibody demonstrated that this PARP isoform cross-linked to MARs as well. The same was observed for well known NMPs like DNA topoisomerase II, the Ku 70 subunit of DNA PK, and C/EBP-β.

Effect of Poly(ADP-Ribosyl)ation Reaction on DNA and Protein Cross-Linking of PARP-1

We examined the effect of the induction of poly(ADP-ribosyl)ation on the capacity of PARP-1 to crosslink to DNA and nuclear proteins. Nuclei were incubated with [^{14}C] NAD in order to induce PARP-1 activation (see "Materials and Methods") after to be treated with NaTT or cDDP: the amount of [^{14}C]poly(ADP-ribose) associated to the TCA-insoluble proteins was determined. In nuclei treated with cDDP before to be incubated with [^{14}C] NAD, we did not find incorporation of radioactivity in the TCA-insoluble material, as an indication that PARP-1 cross-linked to DNA was inactive (data not shown). Vice versa, in nuclei treated with NaTT after to be incubated with [^{14}C] NAD we found TCA-insoluble radioactivity associated to all the nuclear fractions that can be isolated following the procedure that allow the isolation of the NM. Table I reports these values as nanomoles of poly(ADP-ribose)/mg of proteins. It can be seen

TABLE I. [^{14}C]Poly(ADP-Ribose) Associated to TCA-Insoluble Proteins in Rat Testis Nuclear Fractions From Sodium Tetrathionate (NaTT) Cross-Linked Nuclei

Fraction	Poly(ADP-ribose) nanomoles/mg of protein
Nuclei	6.73 ± 2.76
DNAse I sensitive chromatin	6.04 ± 2.44
DNAse I resistant chromatin	3.79 ± 1.86
Nuclear matrix (NM)	13.02 ± 4.56
Internal nuclear matrix (INM)	15.08 ± 5.53
Nuclear matrix proteins (NMPs)	4.69 ± 0.83

that such a value is higher in NM compared to nuclei and to the chromatin fractions isolated by DNase I digestion and 0.5 M $(NH_4)_2SO_4$ extraction. Moreover, we found a high specific radioactivity associated to the INM, as an indication that this fraction is a poly(ADP-ribosyl)ation site.

Next, we looked to the auto-modification level of PARP-1 in NaTT cross-linked nuclei, on the light of our previous results showing that this is the main reaction that does occur in rat testis NM [Quesada et al., 2000]. Silver stained SDS–PAGE pattern is shown as a control of a comparable loading onto the gel (Fig. 4A). Figure 4B shows the results of Western blotting analysis with an anti-PARP-1 antibody. In the NM samples from [^{14}C]NAD incubated nuclei the density of the immunoreactive band in correspondence to the 116 kDa MW of the native form of PARP-1 is greatly reduced, as an indication of an extensive auto-modification of the enzyme. Moreover, densitometric analysis of the NM sample from nuclei subjected to NaTT cross-linking after [^{14}C]NAD incubation revealed that the PARP-1 immunoreactive band represents ~20% of that in control NM, showing that the cross-linking reaction occurs also on auto-modified PARP-1.

Western blotting analysis with an anti-pADPR antibody (Fig. 4C) showed a broad band over the 250 kDa MW marker that can be identified as the auto-modified PARP-1. The higher density of that band in the sample from [^{14}C] NAD incubated nuclei before NaTT treatment, confirmed that protein–protein cross-linking of PARP-1 is compatible with its auto-modification.

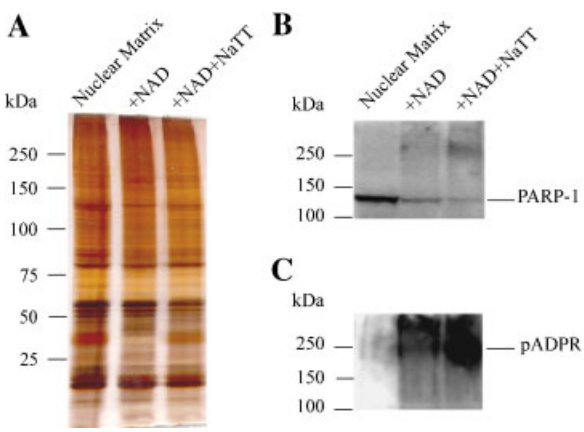

Fig. 4. Western blotting analysis of poly(ADP-ribosyl)ation reaction in rat testis NM (50 μg of proteins) cross-linked or not with 1 mM NaTT after 5–15% SDS–PAGE in the presence of β-MeEtOH. **A**: Silver-staining of the SDS-gel; (**B**) immuno-staining with polyclonal antibody anti-PARP-1 (diluted 1:2,000); (**C**) immuno-staining with monoclonal antibody anti poly(ADP-ribose) (pADPR) (diluted 1:250). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

By inducing protein–protein and DNA–protein cross-linking we confirmed the tendency of PARP-1 to interact with other nuclear components, as a characteristic inherent to its mechanism of action.

It has been reported that PARP-1 physically interacts with a number of nuclear proteins (i.e., XRCC-1, OCT-1, YY-1, NF-kB), therefore modulating their function [Bürkle, 2001; Hassa et al., 2001; Kraus and Lis, 2003]. We show in this article that these interactions can be stabilized by cross-linking reaction with NaTT. In particular, we tested PARP-1 and -2 enzymes that is know to form either homo- and heterodimers and both bind to XRCC-1 [Schreiber et al., 2002]. We observed a retardation of their electrophoretic mobility after treatment with NaTT that suggests their participation to protein complexes not necessary with their selves but also with other nuclear proteins. We found the same for the transcriptional factor C/EBP-β that acts as a master regulator of many cellular responses [Ramji and Foka, 2002]. It has been reported that C/EBP-β is associated to the NM [Uskoković et al., 2002]. Instead, the recombination protein RAD-51 was not cross-linked by NaTT possibly because of its nucleoplasmic localization [Haaf et al., 1995].

In the light of previous evidences [Quesada et al., 2000], we used cross-linking with NaTT to investigate the association of PARP-1 with the NM. By a comparison of the NM isolated from nuclei treated or not with NaTT we observed that cross-linking reaction determined an increment of PARP-1 and C/EBP-β amount, compared to lamin B, as an indication that the induction of disulphide bonds allow the stabilization of NM components functionally related to this fraction. Moreover, we show for the first time the association with the NM of PARP-2 which level in germinal cells is much lower (30%) than PARP-1 [Di Meglio et al., 2004]. The amount of this PARP isoform in the NM was not apparently increased by the NaTT cross-linking. However, the low amount of this PARP

isoform may make difficult a precise quantification of the immunoreactive bands.

Moreover, protein–protein cross-linking reaction allowed us to better determine the localization of PARP-1 in NM. We found that PARP-1 partitioned with the INM isolated by the combined action of nucleases digestion and high salt treatment according with Stuurman et al. [1990]. We observed an even higher amount of PARP-1 in the fraction of NMPs solubilized by treatment with 8 M urea in reducing conditions, that is known to disassemble intermediate filaments of the lamina [Davie et al., 1998]. Interestingly, PARP-1 partitioning resembles that of C/EBP-β and behaves then like a protein functionally associated to the NM.

To ascertain that PARP-1 interacts specifically with NM components we investigated the PARP-1 interaction with DNA MARs. To do this, we first induced DNA–protein cross-linking in nuclei by the use of cDDP that results in the cross-linking of proteins to DNA in situ. Most proteins cross-linked to DNA with cDDP are NMPs like HDAC and CTCF [Dunn et al., 2003]. The PARP-1 associated to all the fractions of the NM isolation procedure, showed an electrophoretic mobility shift, as a consequence of cDDP treatment, that confirmed that PARP-1 is almost totally bound to DNA in nuclei. Moreover, a high amount of such enzyme seemed to interact with MARs. To ascertain this, we determined the presence of PARP-1 in NM fractions after reversion with thiourea of DNA–protein cDDP cross-linking. The same experiment done for PARP-2 confirmed the association of this isoform of PARP with the NM. The small highly basic DNA binding domain contained in the N-terminal portion of the protein can be responsible of interaction with MARs [Schreiber et al., 2002]. The same was observed for other proteins like DNA topoisomerase II and the Ku 70 subunit of DNA PK, that is known to interact with MARs [Chen and Longo, 1996; Galande and Kohwi-Shigematsu, 1999], suggesting that these proteins co-localize with PARP-1 and PARP-2 in these chromatin regions.

We then wondered whether the interaction of PARP-1 to NM would be influenced by the functional state of the enzyme. We observed that PARP-1 covalently linked to MARs by cDDP was inactive as expected. Moreover, we found a high amount of auto-modified PARP-1 in NM after NaTT cross-linking of [^{14}C] NAD^+ incubated nuclei that showed that pADPR contributes to the association of PARP-1 to NMPs. This result can be explained by the non-covalent interaction of the polymer with NMPs [Gagnè et al., 2003].

Moreover, these evidences obtained in rat testis, that contain different types of germinal cells, can be related to previous data that showed higher levels of PARP-1, PARP-2, and PARG enzymes in primary spermatocytes undergoing the meiotic division [Di Meglio et al., 2003, 2004].

In conclusion, our findings confirmed that the NM and the MARs can be seen as a fraction greatly enriched in nuclear proteins, like transcription factors (i.e., C/EBP-β) and enzymes (DNA Topo II, DNA PK) PARP-1 and -2 included. This suggests that PARPs are involved in gene expression control by the NM by their capacity to interact with either DNA and NMPs. We found that such an interaction can be mediated by poly(ADP-ribose) linked to the auto-modified form of PARP-1 associated with the NM. This would confirm that PARP(s) activity is requested for the gene expression coordinated at the NM level. Indeed, this evidence is relevant considering that NMPs are now identified as new biomarkers for screening of early tumor stage.

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