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Regulation of the Expression or Recruitment of Components of the DNA Synthesome by Poly(ADP-Ribose) Polymerase†

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ABSTRACT: Poly(ADP-ribose) polymerase (PARP) is a component of the multiprotein DNA replication complex (MRC, DNA synthesome) that catalyzes replication of viral DNA in vitro. PARP poly(ADP-ribosyl)ates 15 of the ∼40 proteins of the MRC, including DNA polymerase α (DNA pol α), DNA topoisomerase I (topo I), and proliferating-cell nuclear antigen (PCNA). Although about equal amounts of MRC-complexed and free forms of PCNA were detected by immunoblot analysis of HeLa cell extracts, only the complexed form was poly(ADP-ribosyl)ated, suggesting that poly(ADP-ribosyl)ation of PCNA may regulate its function within the MRC. NAD inhibited the activity of DNA pol δ in the MRC in a dose-dependent manner, whereas the PARP inhibitor, 3-AB, reversed this inhibitory effect. The roles of PARP in modulating the composition and enzyme activities of the DNA synthesome were further investigated by characterizing the complex purified from 3T3-L1 cells before and 24 h after induction of a round of DNA replication required for differentiation of these cells; at the latter time point, ∼95% of the cells are in S phase and exhibit a transient peak of PARP expression. The MRC was also purified from similarly treated 3T3-L1 cells depleted of PARP by antisense RNA expression; these cells do not undergo DNA replication nor terminal differentiation. Both PARP protein and activity and essentially all of the DNA pol α and δ activities exclusively cosedimented with the MRC fractions from S phase control cells, and were not detected in the MRC fractions from PARP-antisense or uninduced control cells. Immunoblot analysis further revealed that, although PCNA and topo I were present in total extracts from both control and PARP-antisense cells, they were present in the MRC fraction only from induced control cells, indicating that PARP may play a role in their assembly into an active DNA synthesome. In contrast, expression of DNA pol α, DNA primase, and RPA was down-regulated in PARP-antisense cells, suggesting that PARP may be involved in the expression of these proteins. Depletion of PARP also prevented induction of the expression of the transcription factor E2F-1, which positively regulates transcription of the DNA pol α and PCNA genes; thus, PARP may be necessary for expression of these genes when quiescent cells are stimulated to proliferate.

Poly(ADP-ribose) polymerase (PARP) catalyzes the covalent attachment of poly(ADP-ribose) chains to a variety of nuclear proteins, with NAD as substrate. Consistent with earlier studies with chemical inhibitors of PARP, depletion of PARP from cells by expression of antisense RNA has shown that the enzyme plays important accessory roles in various nuclear processes that involve rejoining of DNA strand breaks. These antisense studies have indicated that depletion of PARP results in a decrease in the initial rate of DNA repair in HeLa cells (Ding et al., 1992) and keratinocytes (Rosenthal et al., 1995), a reduction in the survival of cells exposed to mutagenic agents, an alteration in chromatin structure, and an increase in gene amplification (Ding and Smulson, 1994). In a related approach, fibroblasts derived from PARP knockout mice exhibit proliferation deficiencies in culture, and thymocytes from these animals show a delayed recovery after exposure to γ-irradiation (Wang et al., 1995). More recently, other PARP knockout mice showed reduced survival after exposure to sublethal doses of ionizing radiation, and splenocytes derived from these animals undergo abnormal apoptosis (de Murcia et al., 1997).

We have previously shown that 3T3-L1 cells expressing PARP antisense RNA do not show a transient peak of PARP expression and activity normally apparent 24 h after exposure to insulin, dexamethasone, and methylisobutylxanthine (inducers of differentiation in this system). Consequently, the PARP-depleted cells fail to differentiate into adipocytes (Simbulan-Rosenthal et al., 1996; Smulson et al., 1995). PARP is apparently required for a necessary round of DNA

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replication that occurs within the first 24 h of differentiation in these cells. Confocal microscopy revealed that, during this early stage of differentiation, when essentially all control cells have entered the S phase of the cell cycle, PARP is localized within distinct intranuclear granular foci that are associated with replication centers (Simbulan-Rosenthal et al., 1996). Furthermore, PARP specifically coimmunoprecipitated with DNA pol α during this time in control cells, but not in PARP-antisense cells.

PARP also exclusively copurifies through a series of centrifugation and chromatography steps with core proteins of a multiprotein DNA replication complex (known as MRC, or DNA synthesome) from HeLa cells and mouse FM3A cells; this complex catalyzes replication of viral DNA in vitro and contains DNA pol α and δ, DNA primase, DNA helicase, DNA ligase, and topoisomerase I and II, as well as accessory proteins such as proliferating-cell nuclear antigen (PCNA), RFC, and RPA. Furthermore, immunoblot analysis of MRC from both these cell types with antibodies to poly(ADP-ribose) (PAR) revealed that 15 of the ~40 component proteins, including DNA pol α, topoisomerase I, and PCNA, were poly(ADP-ribosyl)ated.

To clarify the role(s) of PARP within the DNA synthesome during the round of DNA replication in the early stages of differentiation in 3T3-L1 cells, we have now purified and characterized replicative complexes from control cells that had entered S phase after induction of differentiation and from cells depleted of PARP by expression of PARP antisense RNA. In the present study, we demonstrate that the DNA polymerase activities of the DNA synthesome from the S-phase control cells were markedly increased relative to uninduced control cells, whereas, the complex from PARP-deficient antisense cells was devoid of any DNA polymerase activities. To further investigate whether the lack of DNA pol α and δ activities in the PARP-antisense cells can be attributed to present but inactive enzymes, their absence from the complex, or a down-regulation of their expression in the cells, immunoblot analysis was performed with antibodies to replicative enzymes or accessory proteins. Interestingly, we show for the first time that PARP may play a role in the recruitment of PCNA and topo I into the DNA synthesome. These two proteins have been shown to be loosely associated with the core proteins of the DNA synthesome and form a so-called initiation complex (Applegren et al., 1995). On the other hand, PARP also appears to play a role in the regulation of the expression of a number of the proteins comprising the tightly associated core proteins of the MRC, such as DNA pol α, DNA primase, and RPA.

MATERIALS AND METHODS

Cells, Vectors, and Transfection. Monolayer cultures of control and PARP antisense 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL), and subcultured every 4 days. The PARP-antisense cell lines were obtained as previously described (Smulson et al., 1995) by transfection of 3T3-L1 preadipocytes with pMAM-As, a 1.1-kb Xho I fragment of murine PARP cDNA subcloned in the antisense orientation into the expression vector pMAM-neo (Clontech) under the control of the mouse mammary tumor virus long terminal repeat, followed by selection of transfectants in medium containing G-418 (400 μg/mL). Expression of PARP antisense RNA was confirmed by RNA, DNA, and immunoblot analysis of control and stably transfected antisense cell lines after incubation with dexamethasone for various times.

Induction of Differentiation. Control and PARP-antisense 3T3-L1 preadipocyte cells were grown to confluence in DMEM supplemented with 10% FBS and then maintained for an additional 2 days, after which differentiation was induced by addition of 0.5 mM methylisobutyl xanthine, 1 μM dexamethasone, and 1.7 μM insulin. This medium was replaced with DMEM containing 10% FBS and 1.7 μM insulin after 48 h, and cells were placed back in regular medium after another 48 h. Terminal differentiation was monitored by washing the cells in phosphate-buffered saline (PBS), followed by fixation for 10 min in PBS containing 3.7% formaldehyde, staining with 0.3% Oil-Red-O dye for triglyceride droplets for 1 h, and observation under a phase-contrast microscope.

Enzyme Assays. At various indicated times after exposure to inducers of differentiation, control and PARP-antisense cells were harvested, they were washed with ice-cold PBS, and duplicate samples were subjected to enzyme assays to measure PARP, DNA pol α, and DNA pol δ activities. Purified MRC fractions from induced and uninduced control and PARP-antisense cells, as well as from HeLa cells, were also assayed for these enzyme activities. For PARP activity assays, incorporation of [^{32}P]NAD into acid-insoluble acceptors was measured at 25 °C for 1 min, with 20 μg protein per determination and triplicate determinations per treatment (Smulson et al., 1995). In vitro DNA pol α activity was assayed by measuring the incorporation of [^{3}H]TTP into DNA for 1 h at 37 °C by scintillation spectroscopy, with activated calf thymus DNA as template as previously described (Simbulan et al., 1993). DNA pol δ activity assays were performed by measuring the incorporation of [^{3}H]TTP into DNA for 1 h at 37 °C, with polydA-dT as template according to published procedures (Syvaoja et al., 1990).

Purification of the MRC from 3T3-L1 and HeLa Cells. Fractionation of cells and purification of the MRC were performed by a series of centrifugation steps and chromatography on two different columns as previously described (Applegren et al., 1995; Malkas et al., 1990; Wu et al., 1994). The replication-competent MRC partitions exclusively with the P4, Q-Sepharose peak, and sucrose gradient peak fractions, which also exhibit peak activities for DNA pol α, DNA pol δ, and PARP.

Immunoprecipitation Protocols. Immunoprecipitation was performed according to procedures described previously (Simbulan et al., 1993). Briefly, equal amounts (10 μg) of purified MRC (SG fraction) were added with 200 μL of EBC buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 0.5% NP-40, and 0.1 TIU of aprotinin), pre-cleared overnight with 10 μL/sample of protein A-Sepharose at 4 °C. After centrifugation, the supernatants were rocked for an hour with 0.5 mL of NET-N buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing anti-PCNA (1 μg of antibody per sample), followed by another incubation for 20 min with 20 μL of protein A-Sepharose in Tris-buffered saline with 10% BSA (1:1). After extensive washing of the beads with NET-N buffer, the immunocomplex bound to the

beads was then separated by SDS−polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunodetected with monoclonal antibody against poly(ADP-ribose), provided by Drs. M. Miwa and T. Sugimura, Japan (1:250) (Kawamitsu et al., 1983).

Immunoblot Analysis with Antibodies to PARP, DNA pol α, DNA Primase, Topoisomerase I, RPA, and PCNA. SDS−polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots were incubated with rabbit polyclonal antibodies to PARP (1:2000 dilution) (Ludwig et al., 1988) and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots were sequentially stripped by incubation for 30 min at 50 °C with a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), reblocked, and reprobed with antibodies to different DNA replication proteins. The monoclonal anti-DNA pol α (purified ascites) was used at 1:250 dilution and recognizes the 180 kDa polypeptide (Spriggs et al., 1992). The monoclonal antibody to RPA (1:500 dilution; recognizes the RPA p70) was kindly provided by Dr. B. Stillman; and the anti-DNA primase antibody (1:500 dilution; detects the DNA primase p58) by Dr. W. Copeland. The antibodies to topo I (1:1000 dilution; reacts with the topo I p100), PCNA (1:1000 dilution; detects the PCNA p36), and E2F-1 (1: 1000; reacts with the E2F p60) were obtained from TopoGEN, Calbiochem, and Santa Cruz Biotech., respectively.

RESULTS

The Complexed Form of PCNA is Poly(ADP-ribosyl)ated. We have previously shown by immunoprecipitation experiments that PCNA is one of the poly(ADP-ribosyl)ated proteins of the HeLa MRC, together with DNA pol α and topo I. To further characterize the poly(ADP-ribosyl)ation state of PCNA in these cells, we subjected various fractions obtained during the purification of the MRC to immunoprecipitation with anti-PCNA and then to immunoblot analysis with antibodies to PCNA and to PAR. Purification of the MRC was performed by fractionation of cells in a series of centrifugation steps, followed by discontinuous gradient centrifugation on a sucrose cushion (P4 and S4 fractions), chromatography on a Q-sepharose column (QS and FT fractions), and the peak fractions subjected to sucrose gradient centrifugation (SG) as previously described (Applegren et al., 1995; Malkas et al., 1990; Wu et al., 1994). The replication-competent MRC partitions exclusively with the P4, QS peak, and SG peak fractions, which also exhibit peak activities for DNA pol α, DNA pol δ, and PARP. Two forms of PCNA were detected: a complexed form that associated with the replication-competent fractions of the MRC, and a free form present in the replication-inactive fractions (Figure 1). Although approximately equal amounts of these two forms of PCNA were detected in the cell, only the complexed form was poly(ADP-ribosyl)ated (Figure 1), indicating that poly(ADP-ribosyl)ation of PCNA in the MRC may play a role in regulating its functions within the MRC.

Because PCNA is required for synthesis of leading strand DNA synthesis by DNA pol δ (Tsurimoto and Stillman, 1991), we next investigated the effects of further poly(ADP-ribosyl)ation (in the presence of NAD) and the PARP inhibitor 3-aminobenzamide (3-AB) on in vitro DNA pol δ activity within the DNA synthesome. NAD inhibited the activity of DNA pol δ in the HeLa MRC (SG fraction) in a dose-dependent manner (Figure 2A), whereas 3-AB reversed the inhibitory effect of 100 μM NAD (Figure 2B). Essentially identical results were obtained when the effects of NAD and 3-AB on DNA pol α activity in the purified HeLa MRC (SG fraction) were assayed (data not shown). This is the first time that PARP or poly(ADP-ribosyl)ation has been shown to affect the activity of DNA pol δ in vitro, suggesting that PARP may play a regulatory role in the MRC by modulating the activity of component MRC replicative enzymes by catalyzing their poly(ADP-ribosyl)ation. As with other acceptor proteins of PARP (Yoshihara et al., 1985; Darby et al., 1985; Ferro and Olivera, 1984; Kasid et al., 1989; Eki and Hurwitz, 1991), further poly(ADP-ribosyl)ation of these enzymes or their cofactors in the presence of increasing NAD may confer a large negative charge which promotes the dissociation of these enzymes from the DNA template-primer, thereby inhibiting their activity.

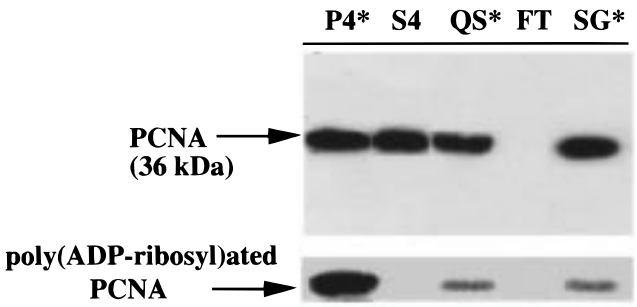

FIGURE 1: Immunoblot analysis with antibodies to PCNA (upper panel) or to PAR (lower panel) of immunoprecipitated fractions obtained during purification of the MRC from HeLa cells. Equal amounts of protein fractions (10 μg) were immunoprecipitated with antibody to PCNA and then subjected to immunoblot analysis with anti-PCNA (1:1000 dilution). Asterisks indicate the replication-competent MRC fractions, including the P4, Q-Sepharose (QS) peak, and sucrose gradient (SG) peak fractions (Applegren et al., 1995; Malkas et al., 1990). The positions of molecular size standards (in kilodaltons) are indicated on the right; the arrow indicates the position of PCNA. The immunoblot in the upper panel was stripped of antibodies and reprobed with monoclonal antibodies to PAR (1:250 dilution).

Effects of Depletion of PARP by Antisense RNA Expression on the Activities of DNA pol α and δ in the MRC of 3T3-L1 Cells. To further elucidate the role(s) of PARP in the MRC, we next investigated the effects of PARP depletion by expression of antisense RNA on the activities of DNA pol α and δ in the MRC (QS peak fractions) purified from 3T3-L1 cells. We have previously shown by flow cytometry that 80% of both 3T3-L1 control and PARP-antisense cells had a predominant G_0-G_1 DNA content prior to induction of differentiation. However, whereas 95% of the control cells had synchronously entered S phase 24 h after induction, 60% of the PARP antisense cells remained blocked at G_0-G_1 and had not entered S phase at this time (Simbulan-Rosenthal et al., 1996). Thus, under these conditions, quiescent control cells are induced to proliferate and go through one round of the cell cycle, but not the PARP-depleted antisense cells. Accordingly, the MRC was purified from 3T3-L1 control and PARP-antisense cells harvested before (0 h) and 24 h

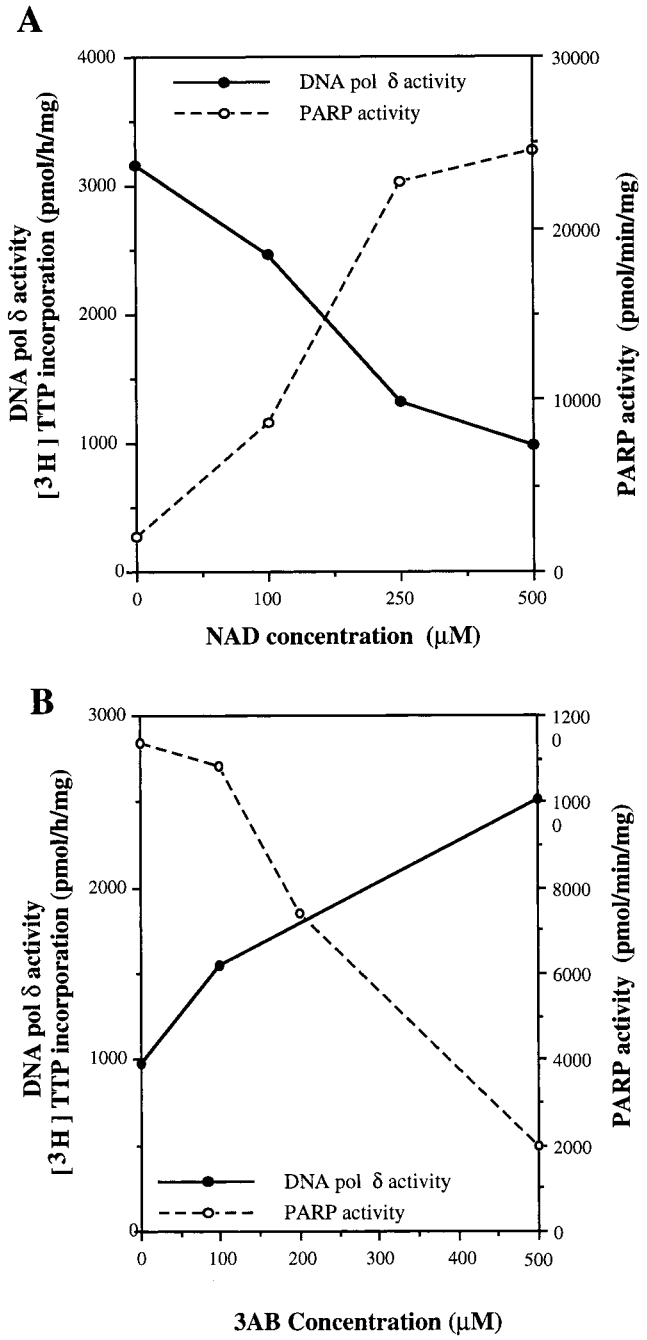

FIGURE 2: Concentration-dependent effects of NAD (A) and 3-AB (B) on DNA pol δ and PARP activities of the purified HeLa cell MRC. PARP and DNA pol δ activities of purified HeLa cell MRC (SG peak fraction) were assayed in the presence of various concentrations of NAD (A), or in the presence of 100 μM NAD and various concentrations of 3-AB (B), as described in Materials and Methods. Data are expressed as pmoles of [^{3}H]dTTP incorporated into DNA per hour per milligram (DNA pol δ) or nanomoles of [^{32}P]NAD per minute per milligram of protein (PARP), and are means of triplicate determinations. Essentially identical results were obtained in three independent experiments.

after induction of differentiation as described in Materials and Methods. The Q-Sepharose peak fractions of MRC purified from the induced control cells which were used in the subsequent experiments were found to be competent to support viral DNA replication in vitro (data not shown).

DNA pol α activity in total cell extracts from uninduced control cells or from the PARP-antisense cells either before or 24 h after induction was only ~15% of that in total

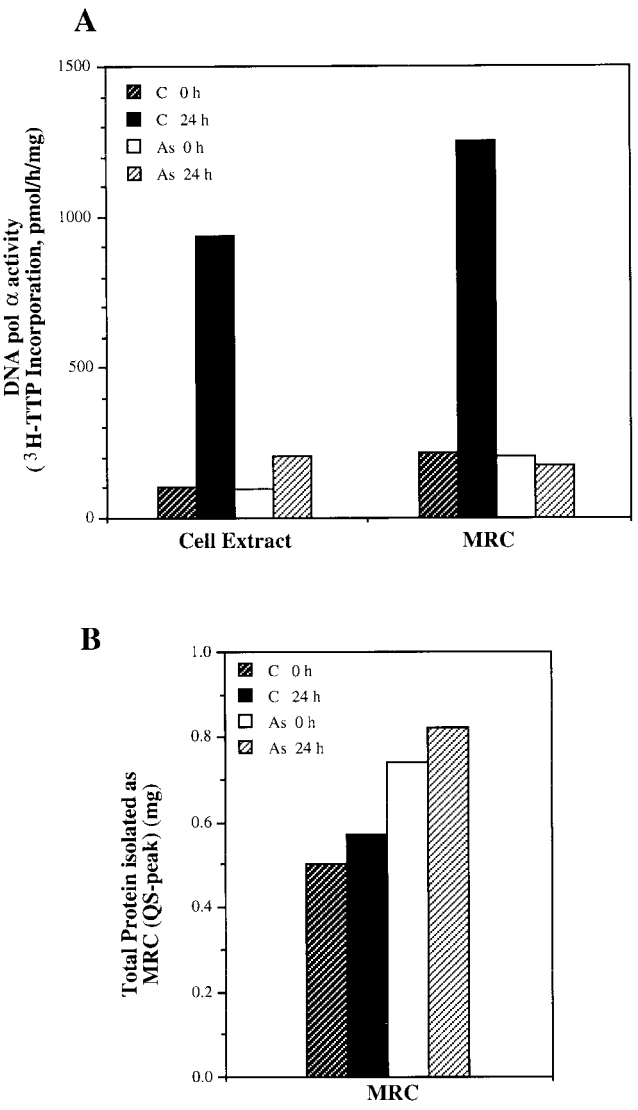

FIGURE 3: Effects of PARP depletion by antisense RNA expression on DNA pol α activity of extracts and MRC fractions of 3T3-L1 cells prepared before or 24 h after induction of differentiation. (A) Total cell extracts and MRC (QS peak) fractions were prepared from 3T3-L1 control (C) and PARP-antisense (As) cells before and 24 h after exposure to inducers of differentiation. DNA pol α activity (pmoles of [^{3}H]dTTP incorporated into DNA per hour per milligram) was assayed as described in Materials and Methods. Data are means of triplicate determinations, and essentially identical results were obtained in two additional experiments. (B) Amounts of protein isolated as MRC (QS peak) from 3T3-L1 (C) and PARP anstisense (As) cells prior to (0 h) and 24 h after induction.

extracts of the S phase control cells (Figure 3). Essentially all of the DNA pol α activity of the replicating control cells was recovered in the MRC fraction; the MRC fractions from PARP-antisense and uninduced control cells contained only ~15% of the DNA pol α activity associated with the MRC fraction of induced control cells (Figure 3A). Comparable amounts of protein were isolated as MRC (QS peak fraction) from the control and antisense cells, before (0 h) and 24 h after induction of differentiation (Figure 3B).

PARP activity assays were performed to confirm that the replicative complex purified from PARP-antisense cells lacked PARP activity. As expected, virtually all PARP activity cosedimented with the replication-competent MRC fractions (P4, QS peak), whereas the replication-inactive fractions (S4, QS flowthrough) contained essentially no

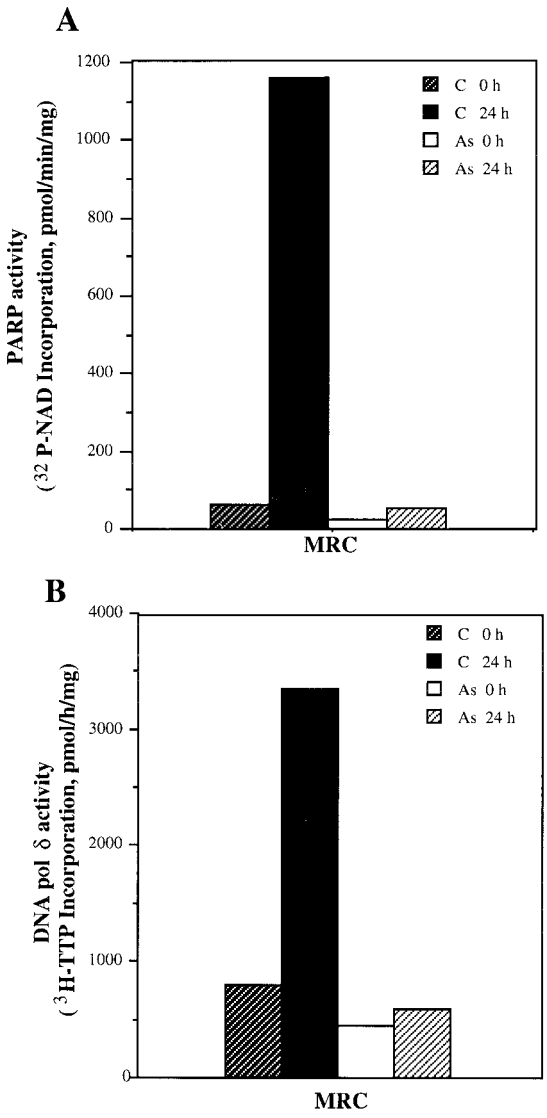

FIGURE 4: Effects of PARP depletion on PARP (A) and DNA pol δ (B) activities in the MRC fractions of 3T3-L1 cells. The MRC (QS peak) fraction was purified from 3T3-L1 control and PARP-antisense cells before and 24 h after induction of differentiation, and subjected to PARP and DNA pol δ activity assays as described in Materials and Methods. Data are means of triplicate determinations, and essentially identical results were obtained in two additional experiments.

PARP activity (data not shown), consistent with PARP being a "core" component of the MRC in these cells. However, as with DNA pol α activity, whereas substantial PARP activity was detected in MRC from control cells in S phase, the MRC fraction from PARP-antisense and uninduced control cells exhibited only ~5% of this activity (Figure 4A). The effect of PARP depletion on DNA pol δ activity of the MRC fraction was virtually identical to that on DNA pol α activity. Only the MRC fraction from replicating control cells contained substantial DNA pol δ activity (Figure 4B). These results are consistent with our earlier results showing that in vivo DNA replication, as assessed by incorporation of bromodeoxyuridine or [^{3}H]thymidine into newly synthesized DNA, occurred only in 3T3-L1 control cells 24 h after induction of differentiation, but not in the PARP-depleted antisense cells. Accordingly, depletion of PARP by antisense RNA expression resulted in a replicative complex devoid of any significant DNA pol α or δ activity.

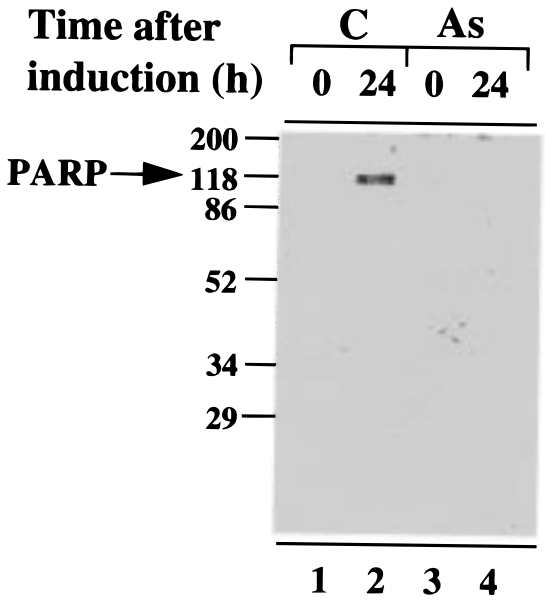

FIGURE 5: Immunoblot analysis with antibody to PARP of MRC fractions purified from 3T3-L1 control and PARP-depleted antisense cells. The MRC (QS peak) fraction was purified from 3T3-L1 control and PARP-antisense cells before and 24 h after induction of differentiation and equal amounts (20 μg) were subjected to immunoblot analysis with antibodies to murine PARP (1:2000 dilution). The positions of molecular size standards (in kilodaltons) are indicated on the left, as is the position of PARP (arrow).

Effects of PARP Depletion on the Protein Composition of the DNA Synthesome of 3T3-L1 Cells. SDS−polyacrylamide gel electrophoresis and silver staining of MRC fractions prepared from control and PARP-antisense cells, before or 24 h after induction of differentiation, revealed that the most prominent difference was the presence of a 116-kDa protein, corresponding to the size of intact PARP, in the fraction from control cells in S phase but not in those from PARP antisense cells or uninduced control cells. Although MRC purified from both control and antisense cells have approximately 35 to 40 protein bands, some proteins are evident only in the control MRC, while other proteins appear to be present in higher amounts in the antisense MRC (data not shown). What these proteins are, whether these bands represent different proteins, or whether some of these bands represent different modified forms of the same proteins, however, remain to be clarified. Consistent with the PARP activity data, immunoblot analysis with polyclonal antibodies to PARP showed that PARP protein was present exclusively in the MRC fraction from control 3T3-L1 cells in S phase, and not in the MRC fractions from PARP-antisense cells or nonreplicating control cells (Figure 5). The polyclonal antibody to PARP used here has previously been shown to react with a 116 kDa protein, corresponding to full-length PARP, and a smaller truncated PARP around 100 kDa which is observed only in murine cell extracts (Ludwig et al., 1988).

We next investigated the effects of PARP depletion on the protein composition of the MRC by immunoblot analysis with antibodies to specific MRC protein components. Immunoblot analysis of total cell extracts revealed that the amounts of PCNA and topo I were markedly increased in control cells exposed to inducers of differentiation (Figure 6). Ponceau S staining for total protein on the same immunoblot confirmed essentially equal protein loading and

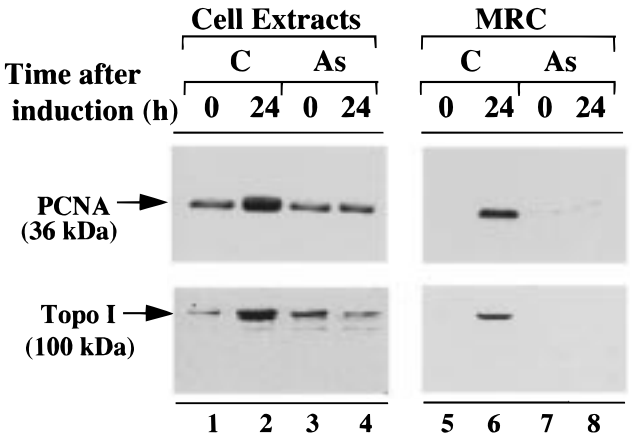

FIGURE 6: Immunoblot analysis of PCNA and topoisomerase I in total cell extracts and MRC fractions prepared from 3T3-L1 control and PARP-depleted antisense cells. Equal amounts (20 μg) of total cell extracts (left panels) and MRC (QS peak) fractions (right panels) from 3T3-L1 control and PARP-antisense cells prepared before and 24 h after induction of differentiation were subjected to immunoblot analysis with antibodies to PCNA (1:1000 dilution) (upper panels) or to DNA topoisomerase I (1:1000 dilution) (lower panels). Arrows indicate the positions of PCNA and topoisomerase I.

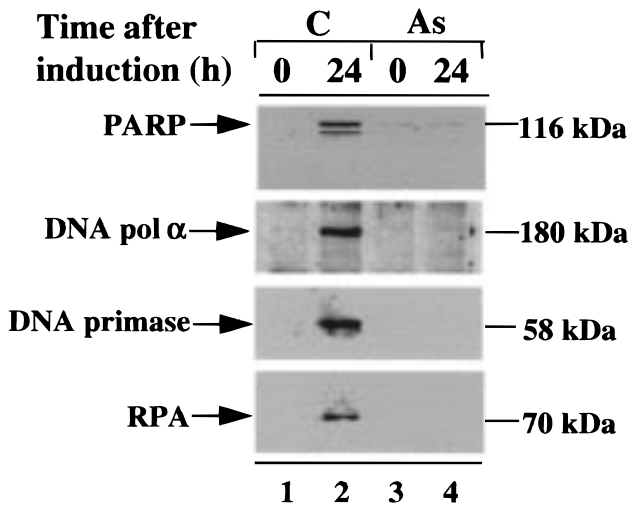

FIGURE 7: Immunoblot analysis of PARP, DNA polα, DNA primase, and RPA in total cell extracts derived from 3T3-L1 control and PARP-depleted antisense cells. Total cell extracts from 3T3-L1 control and PARP-antisense cells were prepared before and 24 h after induction of differentiation and equal amounts (20 μg protein) were subjected to immunoblot analysis with antibodies to PARP, DNA pol α, DNA primase, or RPA. Arrows indicate the positions of the various proteins, and their molecular sizes (in kilodaltons) are indicated on the right.

transfer among lanes (data not shown). It is unclear why topo I was detected in the uninduced PARP-antisense cells, but not in the uninduced control cells; nevertheless, both PCNA and topo I were present in antisense cells (Figure 6). However, the two proteins appeared to be present in antisense cells and uninduced control cells only in the free, uncomplexed form, because they were detected in the MRC fraction only from replicating control cells. These results suggest that these replication proteins are not recruited into the complex in PARP-depleted antisense cells and that PARP may play a role in their assembly into the DNA synthesome.

Immunoblot analysis of total cell extracts with antibodies to other component replicative proteins of the MRC such as DNA pol α, DNA primase, and RPA revealed a significant induction of each of these proteins on exposure of control cells to inducers of differentiation, but not in the PARP antisense cells (Figure 7). These results indicate that PARP and/or poly(ADP-ribosyl)ation may play a role in the regulation of expression of these MRC components when quiescent cells are induced to proliferate. Since there was no effect of PARP depletion on the expression of topo I in these cells (Figure 6), the lack of any DNA pol α, DNA primase, and RPA gene expression could not be attributed to a general inability of the PARP-depleted cells to undergo protein or RNA synthesis.

Effects of PARP Depletion by Antisense RNA Expression on the Expression of E2F-1, a Transcription Factor Implicated in the Transcriptional Regulation of the DNA pol α *Gene*. We next investigated the effect of PARP depletion on the abundance of E2F-1, a transcription factor that positively regulates the transcription of several gene products required for DNA replication and cell growth, including DNA pol α, PCNA, dihydrofolate reductase, thymidine kinase, c-MYC, c-MYB, cyclin D, and cyclin E (Blake and Azizkhan, 1989; DeGregori et al., 1995; Nevins, 1992; Pearson et al., 1991; Slansky et al., 1993). Immunoblot analysis of total cell extracts revealed that, whereas control cells exhibited a marked increase in the expression of E2F-1 as early as 1 h after induction of differentiation, consistent with the fact that the E2F-1 gene is an early-response gene (Johnson et al., 1994), PARP-depleted antisense cells contained negligible amounts of E2F-1 during the 24 h exposure to inducers of differentiation (Figure 8). The induction of both DNA pol α and PCNA in control cells occurred after that of E2F-1, consistent with their being encoded by late-response genes (Pearson et al., 1991). These results indicate that PARP may regulate the expression of DNA pol α and PCNA genes during early S-phase indirectly by affecting the expression of the transcriptional factor, E2F-1, which in turn can regulate the transcription of both the DNA pol α and PCNA genes, as well as the E2F-1 gene itself.

DISCUSSION

We had previously shown that PARP depletion by expression of antisense RNA inhibits the differentiation of 3T3-L1 preadipocytes, including the differentiation-linked round of DNA replication (Smulson et al., 1995). The requirement for DNA replication prior to differentiation is thought to reflect a need to reconfigure chromatin in order to set and change committed patterns of gene expression (Villarreal, 1991). Differentiation of both 3T3-L1 cells (Smulson et al., 1995) and Friend erythroleukemia cells (Spriggs et al., 1992) is prevented by blocking the associated DNA replication at the early stages of this process. Thus, the failure of PARP-depleted 3T3-L1 cells to undergo terminal differentiation into adipocytes is likely attributable to their inability to undergo replication in the early stages of differentiation, indicating that PARP plays a role in this replication.

The roles of PARP have also been examined by gene disruption in PARP knockout mice. While certain strains of PARP knockout mice are viable and fertile, primary fibroblasts derived from these animals exhibit proliferation deficiencies in culture (de Murcia et al., 1997; Wang et al., 1995). Although both DNA replication and differentiation

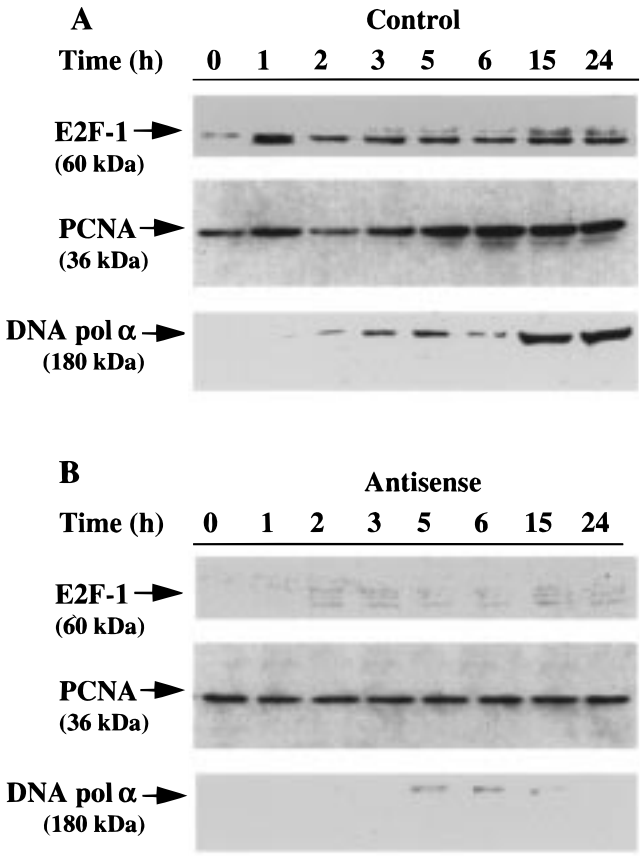

FIGURE 8: Time courses of the expression of DNA pol α, PCNA, and E2F-1 in 3T3-L1 control and PARP-antisense cells after exposure to inducers of differentiation. 3T3-L1 control and PARP-antisense cells were exposed to inducers of differentiation and harvested at the indicated times. Total cell extracts were then prepared and subjected to immunoblot analysis with antibodies to DNA pol α, PCNA, or E2F-1.

must be occurring in these animals, in the absence of PARP, apparently, in clonal cultured cell systems, PARP plays auxiliary roles in these processes, and isolated cell systems show more profound effects due to the lack of PARP that are not apparent in the animals.

We have also shown previously that PARP is tightly associated with the core proteins of the MRC purified from HeLa and FM3A cells (Simbulan-Rosenthal et al., 1996). These purified MRCs support viral DNA replication in vitro and migrates as discrete, high molecular weight complexes on native polyacrylamide gel electrophoresis (Tom et al., 1996). PARP has been thought to play a regulatory role within these complexes because it is capable of modulating the catalytic activity of some of the replicative enzymes or factors either by directly associating with them [DNA pol α (Simbulan et al., 1993)] or by catalyzing their poly(ADP-ribosyl)ation [DNA pol α (Yoshihara et al., 1985), and DNA topoisomerase I and II (Darby et al., 1985; Ferro and Olivera, 1984; Kasid et al., 1989), and RPA (Eki and Hurwitz, 1991)]. In most instances, poly(ADP-ribosyl)ation results in a reduction in enzyme activity of the modified protein, presumably because of a marked decrease in DNA-binding affinity caused by electrostatic repulsion between DNA and PAR.

About 15 of the ~40 polypeptides of the MRC including DNA pol α, topo I, and PCNA, were shown to be poly-(ADP-ribosyl)ated by immunoblot analysis to PAR (Simbulan-Rosenthal et al., 1996). We have now shown that, although there are approximately equal amounts of MRC-complexed and free forms of PCNA in HeLa cells, only the complexed form is poly(ADP-ribosyl)ated (Figure 1), suggesting that such modification may regulate its activity within the complex. The modified, complexed form of PCNA may correspond to the ~35% of total cellular PCNA previously shown to be associated with replication foci during the peak of S phase (Morris and Mathews, 1989).

DNA pol α-primase synthesizes RNA primers and Okazaki fragments required for initiation of the lagging strand, while DNA pol δ mediates leading and lagging strand DNA synthesis during the elongation phase of DNA replication (Waga and Stillman, 1994). PCNA is required for DNA pol δ-mediated synthesis of the leading strand (Tsurimoto and Stillman, 1991). Consistent with previous studies with other purified enzymes, poly(ADP-ribosyl)ation inhibited the activities of DNA pol α and DNA pol δ within the MRC purified from HeLa cells in a manner that was dependent on NAD concentration and sensitive to 3-AB. Thus, poly-(ADP-ribosyl)ation may regulate the catalytic activities of these enzymes within the replication complex.

We also purified the MRC from 3T3-L1 cells harvested before and 24 h after exposure to inducers of DNA replication and differentiation; at the latter time point, ~95% of control cells are in S phase and exhibit a transient peak of PARP expression. Only the MRC fraction from S phase control cells, not those from uninduced control cells or from PARP-antisense cells, contained PARP protein and activity as well as DNA pol α and δ activities (Figures 3 and 4). The observation that the MRC from PARP-depleted antisense cells do not have any DNA pol α and δ activities indicated three possibilities: (i) PARP may be essential for the functions of these replicative enzymes within the complex (they are present but inactive in the PARP-depleted antisense MRC); (ii) PARP may play a role in its assembly into the complex (they are present in total cell extracts, but not in the replicative complex); or (iii) PARP may be implicated in regulation of the expression of the genes for these proteins during entry into S phase (they are not present in cell extracts of the PARP-depleted antisense cells). Thus, the effect of PARP-depletion by antisense RNA expression on the protein composition of the complex was next investigated by immunoblot analyses using antibodies to MRC proteins which were available to us, i.e., PARP, DNA pol α, DNA primase, PCNA, topo I, and RPA.

PCNA and topo I were present in the MRC fraction only from S phase control cells, although they were both detected in total cell extracts from control and PARP-antisense cells exposed or not to inducers of differentiation (Figure 6). These results indicate that PARP may play a role in the assembly of PCNA and topo I into the DNA synthesome during entry into S phase. The mechanism by which this occurs, however, remains unclear. PARP has previously been shown by immunoprecipitation experiments to physically associate with DNA pol α in vitro (Simbulan et al., 1993) and in vivo (Smulson et al., 1995), as well as with topo I (Ferro et al., 1983). This physical association with other proteins may represent a mechanism by which PARP can recruit certain proteins into the replication complex. Whether poly(ADP-ribosyl)ation regulates recruitment of proteins to the MRC remains to be elucidated since it is still unclear whether

modification of component proteins occurs prior to, during, or after association with the MRC.

DNA pol α /DNA primase form a complex of four subunits, the largest of which is the catalytic subunit (~180 kDa) (Wong et al., 1986), and the two smallest subunits comprise the DNA primase (~58- and ~48 kDa) (Bambara and Jessee, 1991). When quiescent cells are stimulated to proliferate, mRNA levels of all four subunits increase simultaneously prior to DNA synthesis; thus, transcription of the genes for DNA pol α and DNA primase are likely regulated by a common mechanism (Miyazawa et al., 1993). Expression of DNA pol α, DNA primase, and RPA were significantly reduced in PARP-depleted antisense cells (Figure 7), suggesting that PARP may be implicated in the expression of the corresponding genes during entry of cells into S phase.

Under the conditions prior to induction of differentiation in this system, cells were stimulated to proliferate from essentially serum-deprived, spatially restricted quiescent cultures. In response to growth stimulation, expression of genes involved in DNA replication has been shown to increase dramatically at late G_1 (Baserga, 1991; Miyazawa et al., 1993), including PCNA, DNA pol α, and DNA primase genes. In mammalian cells, the transcription factor E2F-1 binds to a specific recognition site (5′-TTTCGCGC) and thereby activates the promoters of several genes that encode proteins required for DNA replication and cell growth, including DNA pol α, dihydrofolate reductase, thymidine kinase, c-MYC, c-MYB, PCNA, cyclin D, and cyclin E (Blake and Azizkhan, 1989; DeGregori et al., 1995; Nevins, 1992; Pearson et al., 1991; Slansky et al., 1993). Transcription of the E2F-1 gene, in turn, is also regulated during the cell cycle (Neuman et al., 1994). Interestingly, depletion of PARP by antisense RNA expression also prevented the increase in the abundance of E2F-1 associated with the early stage of differentiation in 3T3-L1 cells (Figure 8). Recently, PARP has also been shown to enhance activator-dependent transcription, presumably by interacting with RNA polymerase II-associated factors (Meisterernst et al., 1997), and a basal transcription factor, TFIIF, was reported to be a highly specific substrate for poly(ADP-ribosyl)ation (Rawling and Alvarez-Gonzalez, 1997). Thus, experiments are now underway to determine whether PARP plays a more direct role in the transcription of DNA pol α, PCNA, and E2F-1 genes by binding to the promoter sequences of the E2F-1 and/or DNA pol α genes during early S phase. Alternatively, since PARP depletion by antisense RNA expression has also been shown to result in significant changes in chromatin structure (Ding et al., 1992), it is also possible that the effects of PARP depletion may be due to indirect alterations in chromatin structure.

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