Selective Isolation of Domains of Chromatin Proximal to Both Carcinogen-induced DNA Damage and Poly-Adenosine Diphosphate-Ribosylation

P. J. Thraves, U. Kasid, and M. E. Smulson

Department of Biochemistry and the Vincent T. Lombardi Cancer Research Center, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007

ABSTRACT

Poly-adenosine diphosphate (ADP)-ribosylation of nuclear proteins has been demonstrated previously to be activated in vivo by the presence of DNA single-strand breaks and has thus been implicated to play an important role in altering chromatin structure during cellular recovery from DNA damage. Based upon these considerations, a novel immuno-technique has been developed, using anti-poly(ADP-ribose) coupled to Sepharose, to enrich for those limited domains of chromatin undergoing poly-ADP-ribosylation. We have used three independent methods to verify the presence of significant levels of single-strand DNA breaks adjacent to polynucleosomes engaged in ADP-ribosylation.

INTRODUCTION

A substantial body of information exists that supports the view that a major role for poly-ADP-ribosylation is the covariant modification of proteins in those domains of chromatin of eukaryotic cells actively engaged in DNA replication and/or recovery from DNA strand breaks (9, 21, 25, 27). Evidence exists that the purified poly(ADP-Rib) polymerase possesses a strong binding affinity to DNA containing single-strand breaks (17). Furthermore, it has been demonstrated that the ability of DNA to support poly(ADP-Rib) synthesis is completely dependent upon the number of phosphodiester breaks contained within the DNA (1). Accordingly poly(ADP-Rib) polymerase activity has been shown to increase with cellular treatments which cause DNA single-strand breaks such as MNU, ionizing radiation, endonucleases, and numerous DNA-alkylating drugs (2, 4, 7, 16, 25, 27). In addition, the use of inhibitors of the polymerase activity following DNA damage has been shown to prevent the fall in NAD levels and to potentiate the persistence of DNA strand breaks (9, 20, 21, 24, 33). However, it should be noted that DNA strand breaks may not be the sole activator for poly-ADP-ribosylation, since in the cellular elevation of polymer has been observed by treatments in the absence of DNA damage (7, 26, 33).

Previous studies on the relationship between DNA damage and poly(ADP-Rib) synthesis have been performed mainly at the cellular level. The aim of this report was to evaluate a method of isolating preferentially poly-ADP-ribosylated oligonucleosomes from the bulk of chromatin. The oligonucleosomes bound to the antibody accounted for essentially 100% of the poly-ADP-ribosylated nucleosomes in the unfractionated chromatin and contained poly-ADP-ribosylated core histones, histone H1 and poly(ADP-Rib) polymerase. We have subsequently used this immunofractionation method to study the relationships between poly-ADP-ribosylation and histone H1 phosphorylation (31), chromatin (15), and histone acetylation (32), and the replicative forms of polyoma minichromosomes (19). Therefore, this procedure appears to offer the potential to isolate nucleosomal domains proximal to this one specific modification region of chromatin. The present study demonstrates the potential of this technique to isolate preferentially oligonucleosomal species adjacent to DNA strand breaks, following treatment in vivo of HeLa cells with an alkylating agent.

MATERIALS AND METHODS

Coupling of anti-poly(ADP-Rib) IgG to cyanogen bromide-activated Sepharose-4B was performed as described previously (14). HeLa cell nuclei were prepared by the method of Sporn et al. (23). Nucleosoluble chromatin was isolated as described previously (6). Under these digestion conditions, 8 to 12% of the chromatin DNA was rendered acid soluble. Poly(ADP-Rib) polymerase activity in nucleosoluble chromatin was estimated as described previously (13), and the ADP-ribosylated nucleosomes were analyzed by electrophoresis in native polyacrylamide gels.

In Vitro Incubation with $^{32}$P]NAD. $^{32}$P]Jara-C-labeled chromatin (approximately 5.0 units) was suspended in 10 mM Tris-HCl, pH 8.0, containing 2 mM dithiothreitol and 5 mM MgCl$_2$. The reactions were initiated by the addition of 0.2 $\mu$Ci of $^{32}$P]NAD containing 10 $\mu$M nonradioactive NAD and incubated at room temperature for 5 min. The reactions were terminated by the addition of nicotinamide to a final concentration of 20 mM. The labeled nucleosomal samples were then passed through small columns of Sephadex G-25 to remove excess unincorporated NAD.

Antibody Column Chromatography. Appropriate amounts of in vitro-labeled $^{32}$P]ADP-ribosylated oligonucleosomes were applied slowly to the column over a 30-min period. Washing was initiated with phosphate-buffered saline, and thirty 1-ml fractions were collected. Bound material was released by the addition of 1.6 $\mu$g KSCN (10 x 1-ml fractions). Following the release of bound material, the column was washed with a further 20 ml, and 100-ml aliquots of the column fractions were placed in 1 ml of 10% trichloroacetic acid-5 $\mu$g pyrophosphate, filtered, and counted. Poly(ADP-Rib) polymer species were isolated and analyzed by gel-electrophoretic analysis as described previously by Butt and Smulson (6).

RESULTS

Several of the experiments below were performed with poly-nucleosomes prepared by micrococcal nuclease digestion of...
nuclei isolated from HeLa cells which had been incubated in the presence or absence of MNU. Oligonucleosomes isolated from HeLa cell nuclei by a brief digestion (i.e., 8 to 12% solubility) with micrococcal nuclease have been shown to have higher specific activities for DNA replication (i.e., [3H]thymidine incorporation) and poly(ADP-Rib) polymerase activity (13). Further, Hough and Smulson (12) have demonstrated recently that this 8 to 12% of total nucleosomes released by micrococcal nuclease are enriched in transcriptionally active species, and they have studied this with respect to the ADP-ribosylation of nuclear proteins. In a previous study by Jump et al. (13), it was shown that poly(ADP-Rib) polymerase has a greater activity in the nuclease-solubilized oligonucleosomes rather than the residual chromatin. Accordingly, since all studies were performed with a small percentage of solubilized chromatin, prior to the evaluation of the anti-poly(ADP-Rib) IgG affinity column as a means of isolating a region of chromatin proximal to the strand breaks, equal A260 aliquots of oligonucleosomes were assayed in vitro with [32P]NAD under standard conditions and electrophoresed in native polyacrylamide gels. Following autoradiography, it was observed that treatment of HeLa cells with MNU stimulated the ADP-ribosylation of the nuclear proteins in the isolated oligonucleosomes (Fig. 1). Under these conditions of enhanced ADP-ribosylation, we have shown recently that poly(ADP-Rib) polymerase itself is autmodified at least 3-fold compared with polymerase from untreated cells and that there is a significant increase in the modification of the core histones and histone H1 in these isolated oligonucleosomes (21, 27).

Immunofractionation of Poly(ADP-Rib) Chromatin. In the current study, we have isolated chromatin regions adjacent and distant to sites of poly-ADP-ribosylation (see below) and assessed the presence of DNA strand breaks in the preparations by 3 methods. Cells were labeled with [3H]thymidine subsequent to in vivo DNA damage with MNU. Alternatively, endogenous 3'-hydroxyl DNA termini were labeled in vivo with [3H]ara-C. Additionally, DNA was extracted from immunofractionated nucleosomes derived from cells treated with MNU and tested directly for internal DNA strand breaks by 2-dimensional electrophoresis. In the immunofractionation procedure on anti-poly(ADP-Rib)-Sepharose, poly(nucleosomes are incubated with [32P]NAD (see Fig. 1) and fractionated on the antibody column (14, 15, 19, 31, 32). Routinely, we have noticed that not all the [32P] acid-insoluble radioactivity binds to the antibody (i.e., unbound fractions in Chart 1), and it was of importance to characterize this labeled unbound material. One possibility was that the polymer chain lengths of those acceptors were very short and, therefore, unrecognized by the antibody. To test this, identical aliquots of oligonucleosomes derived from both control and MNU-treated HeLa cells were incubated with a low (10 \( \mu \)M) concentration of [32P]NAD and passed separately over the antibody column as described in detail below (i.e., Fig. 2). Following immunofractionation in which approximately 70% of acid precipitable chromatin was bound to the column, the labeled poly(ADP-Rib)-Sepharose chains from both the bound and unbound chromatin were cleaved from the nuclear protein acceptors by treatment with weak alkali, 100 mM Tris, pH 10.0, and then analyzed by electrophoresis in 20% polyacrylamide-8 M urea denaturing gels (Fig. 2) as described previously (27). The results of such an analysis showed that the polymer chains in the bound chromatin samples were much longer than those of the unbound nucleosomes and that the polymers from the MNU-treated nucleosomes had a higher specific activity (i.e., a greater incorporation of radiolabel). Thus, much of the unbound ADP-ribosylated material to be described in our studies probably represents acceptors with shorter polymer chains. The data above suggest that the nucleosomes from MNU-treated cells used in this analysis existed in a more highly ADP-ribosylated state and contained slightly longer polymer chain species.

[3H]Thymidine Incorporation after DNA Damage. Three independent methods were followed to study the association of poly-ADP-ribosylated domains of chromatin with DNA strand breaks. It had been shown previously, using HeLa cells synchronized in mid-S phase, that maximal specific activity of the polymerase itself and the modification of the nuclear protein acceptors for poly(ADP-Rib) are preferentially associated with regions of chromatin contiguous with the DNA replicating (and repair) fork (13). Thus, in the first method, unscheduled DNA synthesis (as an indirect measure of patched regions) was estimated by the use of a 10-min pulse of [3H]thymidine after MNU treatment in...
it was observed that greater than two-thirds of the ADP-ribosylated material was bound to the column (Chart 1, A and B). Unbound material was shown above to consist predominantly of short-chained ADP-ribosylated material. With the control nucleosomes (Chart 1), a negligible amount of the [3H]thymidine incorporation was retained by the column. In contrast, with the nucleosomes derived from MNU-treated cells, over 30% of the [3H]thymidine was observed to be retained by the anti-poly(ADP-Rib)-column.

To test the specificity of this affinity binding, an identical aliquot of [32P]ADP-ribosylated nucleosomes from MNU-treated cells was chromatographed with a similarly prepared column containing preimmune IgG coupled to Sepharose (Chart 1). The majority of the acid-insoluble 32P- or 3H-labeled material remained unbound to the column and eluted with the bulk of the nucleosomes. Only a negligible amount of either radiolabel was eluted with KSCN. A similar elution profile was observed with [32P]ADP-

the presence of hydroxyurea. We used plateau-phase cells (i.e., nondividing) and a relatively high concentration of hydroxyurea to ensure effective inhibition of semiconservative DNA replication. During the treatment of the cells and the subsequent isolation of nuclei and preparation of chromatin, it was noted that there was a consistently greater than 2-fold increase in the specific activity of [3H]thymidine label in the isolated oligonucleosomes compared to the nuclear or cellular incorporation. In addition, the thymidine incorporation was at least 2-fold higher in preparations derived from cells incubated with the DNA strand breaking agent (data not shown).

Having observed that the isolated chromatin from MNU-treated cells was enriched in both thymidine incorporation and had a higher poly(ADP-Rib) polymerase activity than the untreated cells, aliquots of either control or treated nucleosomes were ADP-ribosylated in vitro with 10 μM [32P]NAD (0.2 μCi) and equilibrated with anti-poly(ADP-Rib) IgG columns as described in detail recently (14). The columns were washed with 10 ml of phosphate-buffered saline to remove unbound material (approximately 80 to 90% of the original chromatin; Ref. 14); bound nucleosomes were eluted with 10 ml of 1.6 M KSCN. Upon chromatography of either control or MNU-treated nucleosomes,

Chart 1. Selective retention of [3H]thymidine-labeled chromatin from MNU-treated cells on anti-poly(ADP-Rib)-IgG-Sepharose. The coupling of anti-poly(ADP-Rib)-IgG to cyanogen bromide-activated Sepharose-4B was performed as described previously (14). [3H]Thymidine-labeled chromatin samples (1.5 AMO units) (see Fig. 1) from either control (B) or MNU-treated (A) were ADP-ribosylated in vitro with 10 μM [32P]NAD (0.2 μCi) as described previously (14). Antibody column chromatography was performed by applying appropriate amounts of the labeled oligonucleosomes to antibody columns over a 30-min period. Washing was initiated with phosphate-buffered saline, and thirty 1-ml fractions were collected. Bound material was released by the addition of 1.6 M KSCN (30 x 1-ml fractions). Following the release of 100-μl aliquots of the column, fractions were placed in 1 ml of 10% trichloroacetic acid-5 mM pyrophosphate, filtered, and counted. A similarly prepared poly-ADP-ribosylated oligonucleosomal sample (C) from MNU-treated cells was analyzed on a preimmune IgG-coupled Sepharose column. [32P]-labeled poly-ADP-ribosylated (○); [3H]thymidine unscheduled DNA synthesis ( (!$)); [3H]Tdr, [3H]thymidine.
Poly(ADP-Rib) AND DNA STRAND BREAKS

At any one time, the regions of chromatin undergoing poly-ADP-ribosylation, as well as DNA replication or repair, only represent a small percentage of the total genome (6, 22). Therefore, a means of selectively isolating such regions would be of great importance in establishing the molecular mechanisms by which poly(ADP-Rib) and DNA strand breaks physically interact.

Association of ara-C Incorporation with Poly-ADP-ribosylated Domains of Chromatin. The in vivo signal for generation for poly-ADP-ribosylated nuclear proteins in various chromatin regions appears to be the presence of single-strand breaks in cellular DNA (1, 9, 16, 21, 25, 27). The major difficulty in studying this in vivo using the current immunofractionation techniques is that repair is rapid and progresses at only a few sites at any given time. Thus, there are few intermediate structures available for study. Accordingly, to obtain an alternate method to indirectly “fingerprint” sites of strand breaks in vivo, we used ara-C to accumulate such intermediates and to investigate the relationship of these truncated regions within the immunofractionated chromatin. Previous studies have indicated that the incorporation of ara-C partially terminates DNA polymerization prior to ligation, thus potentiating the existence of strand breaks (5, 8). The incorporation of [3H]ara-C into chromatin was determined by first treating stationary-phase HeLa cells in normal media with [3H]-ara-C, to give a final concentration of 1 mw ara-C. In this study, we intentionally did not subject the cells to alkylation DNA damage prior to ara-C treatment, in order to minimize the number of single-stranded regions of DNA occurring during the experimental procedure. Accordingly, the study assessed the incorporation of [3H]ara-C into endogenous repair patches in HeLa cells maintained in stationary phase. Berger et al. (3) have shown which levels of poly(ADP-Rib) synthesis occur concomitant with increases in endogenous DNA strand breaks during plateau phase, at which point cells are in G1 phase. Further, treatment of cells with ara-C was also shown to arrest cycling cells in G1, leading to an increase in polymer synthesis.

Micrococcal nuclease soluble chromatin was prepared from these cells, and the various polynucleosomal chain sizes separated on linear sucrose gradients (6). Electrophoresis of these particles indicated that the ara-C was incorporated into nucleosomes of various chain sizes (data not shown). Polynucleosomes of chain size approximately 8N were incubated in vitro with [32P]-NAD and immunofractionated as in Chart 1. Essentially two-thirds of the poly-ADP-ribosylated chromatin was retained by the column; however, this represented only 8% of the total chromatin applied (Table 1). Approximately, 62% of the acid-insoluble [3H]-ara-C incorporation was associated with the unbound chromatin accounting for 92% of the original A260 nm units applied. The two-thirds of the poly-ADP-ribosylated material retained accounted for 38% of the total [3H]ara-C incorporation. The specific activity of [3H]ara-C (cpm/A260 unit) was calculated for both bound and unbound chromatin and indicated a significant enrichment of [3H]ara-C incorporation within the bound poly-ADP-ribosylated fractions, thus confirming the previous observation with [3H]thymidine incorporation (Chart 1).

Direct Analysis of DNA Strand Breaks Associated with Poly-ADP-ribosylation Chromatin. In an earlier study, we used 2-dimensional electrophoresis to demonstrate the existence of internal-strand DNA breaks in immunofractionated chromatin from exponentially dividing cells (14). In the current study, we have extended this method to investigate the DNA adjacent to poly-ADP-ribosylated sites in chromatin derived from cells treated with an alkylating agent. In an experiment performed similarly to that described in Chart 1 (with the exception that radioactive thymidine was not used, DNA was extracted from both bound and unbound column fractions and was treated with alkaline phosphatase and end-labeled with [32P]ATP and polynucleotide kinase. The 32P-labeled DNA was subsequently analyzed for strand breaks on a native agarose gel electrophoresed in one direction and an alkaline-denaturing agarose gel run at right angles to the first dimension (14). Ethidium bromide staining of the first dimension (Fig. 3A) shown a typical nucleosome repeat ladder of DNA sizes of approximately 200 to 1000 base pairs in the unbound chromatin. It is of note that, in the unbound chromatin (the majority of the DNA), the migration of these DNA repeat sizes in the denaturing gel (second dimension) corresponded essentially to unbroken DNA. The autoradiogram and ethidium bromide stain of this gel corresponded closely (Fig. 3A). Nucleosomal DNA containing strand breaks would be expected to migrate faster, i.e., below the diagonal in the second dimension denaturing gel. It was seen that a negligible amount of the 32P-labeled DNA from the unbound chromatin migrated in this manner, suggesting that these nucleosomes contained few, if any, MNU-induced strand breaks (perhaps that their repair had already occurred in vivo). Very little DNA was obtained from the bound nucleosomes in the MNU experiments (Fig. 3B; Table 1). However, in contrast to the unbound nucleosomes, these fractions, although migrating to the same distance in the first dimension, migrated further in the second dimension, thus indicating the presence of an increased number of DNA strand breaks in the bound chromatin.

These results, coupled with our earlier experiments, have confirmed numerous in vivo and in vitro experiments by directly showing the association of poly-ADP-ribosylated chromatin domains with regions of patched or single-stranded DNA. This immunofractionation methodology therefore should be useful in their isolation from cells treated in vivo.

DISCUSSION

An initial observation by Whish et al. (30) showed that treatment of eukaryotic cells with the antitumor antibiotic Streptozotocin resulted in a stimulation of poly-ADP-ribose polymerase activity. Following this, studies performed in our laboratory showed that Streptozotocin and MNU stimulated poly-ADP-ribose polymerase activity in isolated nuclei (24), and further, this

| Table 1 Association in vivo of [3H]ara-C with ADP-ribosylated chromatin |
| HeLa Sz cells were resuspended in fresh medium at a concentration of 8 x 106/ml and labeled with [3H]ara-C (250 µCi/250 µl) at 8 µCi/ml for 10 min. The cells were then harvested and nuclei purified, and micrococcal nuclease-soluble chromatin was prepared and immunofractionated as described in Chart 1. |
| Fraction | [3H]Poly(ADP-Rib) (cpm) | [3H]ara-C (cpm) | A260 nm | Specific activity of [3H]ara-C |
| Unfractionated chromatin | 1843 | 3061 | 4.85 | 631 |
| Unbound | 625 (34) | 1903 (62) | 4.47 (92) | 425 |
| Bound | 1218 (66) | 1156 (38) | 0.38 (8) | 3047 |

* Calculated from a separate unlabeled column run, performed in parallel. Numbers in parentheses, percentage.
Fig. 3. Influence of treatment in vivo with MNU on internal DNA strand breaks in nucleosomes adjacent to poly-ADP-ribosylated chromatin. HeLa cells were incubated with MNU as described in the legend to Fig. 1, and polynucleosomes immunofractionated on anti-poly(ADP-Rib) Sepharose columns as described previously in the legends to Chart 1, Fig. 2, and Table 1. DNA was extracted from the unbound and bound nucleosomes and labeled by using [3H]ATP and polynucleotide kinase (14). DNA samples were subjected to nondenaturing 1.6% agarose gel electrophoresis (first dimension) and a denaturing gel electrophoresis in a second dimension as described previously (14). The gels were stained with ethidium bromide prior to autoradiography. Arrows, direction of migration of DNA during electrophoresis. A, unbound oligonucleosomes; B, bound oligonucleosomes of DNA.

increase could be observed by the modification of nuclear proteins acceptors at the oligonucleosomal level of isolated chromatin (24, 27).

At any one time, the regions of chromatin undergoing DNA replication or repair probably only represent a small percentage of the total genome. Therefore, a means of selectively isolating such regions would be of great importance in establishing the molecular mechanisms by which poly(ADP-Rib) and strand breaks physically interact.

In a previous study, the use of Sepharose-bound antipoly(ADP-Rib) antibody resulted in a selective isolation of oligonucleosomes species proximal to sites of extensive poly-ADP-ribosylation with chromatin. The endogenous levels and distribution of poly(ADP-ribose) are small, representing only 1 to 5% of the total potential histone and nonhistone protein acceptors at any one time (6, 13, 22). Furthermore, the oligomeric DNA species in such affinity-bound nucleosomes (albeit, in the earlier study, derived from nondamaged cells) migrate as an aggregate and faster in a denaturing 2-dimensional agarose gel system, than the corresponding DNA from the unbound chromatin. This suggests that chromatin-containing strand breaks might exist in the nucleosomal bound to the affinity column.

In this study, we have observed an enhancement (2-fold) and a specific retention of hyper-ADP-ribosylated, in vivo [3H]thymidine-labeled chromatin, following treatment with the carcinogen MNU. Additionally, the incorporation of the chain terminator ara-C (5, 8) was also enriched in the poly-ADP-ribosylated nucleosomes. Most of these latter nucleosomes possessed a greater number of internal DNA strand breaks. Further, the retention of these chromatin regions to the antibody column is due to the increased synthesis of longer polymer chains (Fig. 2) on the protein acceptors of these chromatin domains (14, 27). Recent studies have implicated the formation of poly(ADP-Rib) on nuclear proteins with the activation of DNA ligation following unscheduled DNA synthesis (17) and the inactivation in vitro of DNA topoisomerase activity (10).

Accordingly, this immunoaffinity methodology offers a means of isolating, for subsequent analysis, the dynamic domains of chromatin undergoing DNA synthesis and/or repair.

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